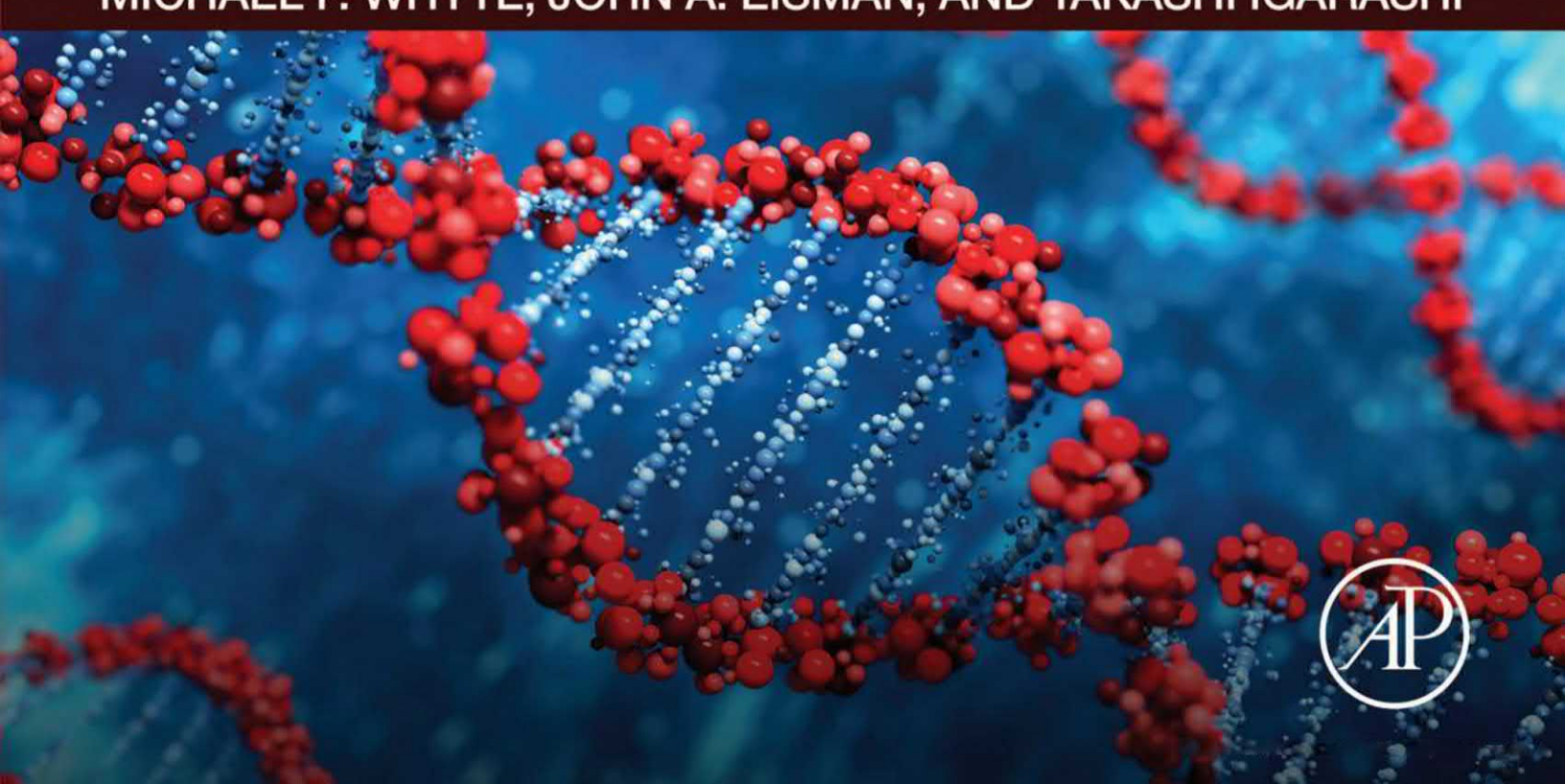
SECOND EDITION

GENETICS OF BONE BIOLOGY AND SKELETAL DISEASE

EDITED BY **RAJESH V. THAKKER**
MICHAEL P. WHYTE, JOHN A. EISMAN, AND TAKASHI IGARASHI



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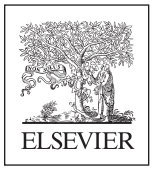

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Preface to the Second Edition

It has been our pleasure to compile the second edition of the *Genetics of Bone Biology and Skeletal Disease*, which contains details of many exciting advances that have occurred since the publication of the first edition in 2013. These past 5 years have seen a rapid pace of fruitful research that has resulted in major achievements particularly in the fields of genome and epigenetic analysis, stem cells, osteoimmunology, osteocyte biology, and translational studies with new therapies. The aim of the second edition is to harness this progress in an easily understandable manner for the reader, and to provide a reference framework that will inspire further investigation and innovation. Thus, the second edition of the *Genetics of Bone Biology and Skeletal Disease* has >20% new chapters, as well as the previous chapters that have been extensively expanded and updated to incorporate these important advances.

The second edition, now containing 43 chapters, is aimed at all students of bone biology and genetics, and with this in mind it includes general introductory chapters concerning genetics and bone biology, followed by more specific disease orientated chapters, which comprehensively summarize the clinical, genetic, diagnostic, molecular genetic, *in vitro* and *in vivo* pathophysiology, and treatment aspects of each disorder. The chapters are organized into five sections that each emphasize particular themes, which are: General background to genetics; General background to bone biology; Disorders of bone and joint, Parathyroid and related disorders; and Vitamin D and renal disorders. The first section "General Background to Genetics" provides an introduction to genetics of skeletal and mineral metabolic diseases, followed by similarly erudite reviews of: epigenetics; genome-wide association studies (GWAS), copy number variation; genomic profiling; functional genomics; mouse models and approaches to generate in vivo models; prospects of gene therapy; pharmacogenomics and pharmacogenetics of osteoporosis with a personalized medicine outlook; and genetic testing and counseling. The second section "General Background to Bone Biology" is specifically devoted to providing an overview of: bone and cartilage biology; bone structure; joint and cartilage biology; osteocyte biology; stem cells; osteoimmunology; integration of endocrine and paracrine influences on bone; the principles of endocrine regulation of bone and fat, with the role of neuronal regulation and energy homeostasis; and the control of mineral and skeletal homeostasis during pregnancy and in the fetus. The third section "Disorders of Bone and Joint" details the advances in the genetics and molecular biology of: the osteoporosis genes identified by GWAS; monogenic forms of osteoporosis; osteoarthritis; Paget's disease; Mendelian disorders of RANKL, OPG, RANK, and NF-KB signaling; skeletal dysplasias and hypophosphatasia; sclerosing bone disorders; fibrodysplasia (myositis) ossificans progressive; thyroid hormone in bone and joint disorders; and extraosseous calcification. The fourth section "Parathyroid and Related Disorders" highlights the central role of the parathyroids in calcium and skeletal homeostasis by reviewing the molecular genetics of: hyperparathyroidism, hypoparathyrodism, pseudohypoparathyroidism, and the McCune–Albright syndrome; disorders of the PTH receptor, its ligands, and downstream effector molecules; disorders of the calcium-sensing receptor; and the multiple endocrine neoplasia MEN syndromes. The fifth section "Vitamin D and Renal Disorders" details the molecular and cellular advances in: genetic disorders of vitamin D synthesis and action; renal phosphate wasting disorders; the Fanconi syndromes, Dent's disease, and Bartter's syndrome; magnesium disorders; and hypercalciuria. Each chapter has been written to provide an authoritative and comprehensive account that can stand alone. Thus, there is inevitably some overlap among the information in some chapters, and this is advantageous, as it provides different viewpoints and interpretations. It is our sincere hope that this second edition will contribute to a better understanding of the complexity at the genetic, cellular, and physiological levels of bone biology and skeletal disorders while guiding and inspiring clinicians and researchers in many related disciplines (including endocrinology, metabolism, nephrology, rheumatology, orthopedics, pediatrics, genetics, biochemistry, physiology, pathology, and pharmacology) to strive to bring improvements for the diagnosis and safe treatment of patients.

The authorship of the second edition has been broadened to include the foremost and internationally outstanding individuals in the field. We are privileged to

have the honor of working with such wonderful people, who have delivered their chapters on time, and remained friends.

Finally, such an endeavor succeeds only with the help of a dedicated and committed team. In particular, we are grateful to Mara Conner, Tari Broderick, and Timothy Bennett at Elsevier, who encouraged us in this venture and who have worked tirelessly with us to bring this highly informative and exciting book to you. We hope you will find this book rewarding and inspiring; we certainly have!

Rajesh V. Thakker
John A. Eisman
Takashi Igarashi
Michael P. Whyte

Preface to the First Edition

Bone and joint disorders are common and represent a major burden on health economics costs. For example, osteoporosis and related fractures are the leading cause of hospital admission in women over the age of 50 years, and >80% of adults over 55 years of age will suffer from osteoarthritis. Treatments of some metabolic disorders have been greatly improved by an increased understanding of bone and joint biology that has been facilitated by advances in unravelling underlying genetic contributions. These interactions between bone and joint biology, physiology and genetics have also greatly enhanced the understanding of normal bone function as well as the molecular pathogenesis of metabolic bone disorders.

The maintenance of bone and mineral metabolism involves a multiorgan system that includes endocrine and neuronal pathways, the kidneys, adipose tissue, gut, vitamin D metabolism and the specialized cells of the bone. These organs and pathways act in a coordinated manner with feedback systems between bone and the internal environment, and characterization of these has greatly clarified the regulation of skeletal biology and the underlying homeostatic mechanisms. These advances in bone biology have been facilitated by the advances in human genetics, such as the sequencing of the human genome. The application of these advances in human genetics has facilitated identification of the genetic abnormalities causing skeletal disease and this, in turn, has advanced our understanding of normal bone biology as well as the development of new therapies.

The purpose of this book is to identify and analyse the genetic basis of bone disorders in humans and to demonstrate the utility of mouse models in furthering the knowledge of mechanisms and evaluations of treatments. The book is aimed at all students of bone biology and genetics and, with this in mind, it includes general introductory chapters on bone and biology, and genetics; and more specific disease orientated chapters, which comprehensively summarize the clinical, genetic, molecular genetic, animal model, functional and molecular pathology, diagnostic, counseling and treatment aspects of each disorder. The book is intended to be comprehensive but readable, and each chapter is relatively brief yet complete in itself. As a consequence, there is some overlap among some of the information in some chapters, and this is advantageous in providing different viewpoints and interpretations.

The book is organised into five sections that each emphasize particular themes, which are: General background to bone biology; General background to genetics; Disorders of bone and joint; Parathyroid and related disorders; and Vitamin D and renal disorders. The first section "General Background to Bone Biology" is specifically devoted to providing an overview of: bone biology and structure; joint and cartilage biology; principles of endocrine regulation of bone and the role of neuronal regulation and energy homeostasis. The second section "General Background to Genetics" reviews the principles and progress in: genome-wide association studies (GWAS); genomic profiling; copy number variation; prospects of gene therapy; pharmacogenomics; genetic testing and counseling; the generation and utilizing of mouse models and how these have helped increase our understanding of mineral and skeletal homeostasis during pregnancy and in the fetus. The third section "Disorders of Bone and Joint" details the advances in the genetics and molecular biology of: monogenic forms of osteoporosis; GWAS of complex forms of osteoporosis; osteoarthritis; Paget's disease; heritable disorders of RANKL, OPG, RANK and NF-κB signaling; skeletal dysplasias, hypophosphatasia; disorders of high bone mass; and fibrodysplasia (myositis) ossificans progressiva. The fourth section "Parathyroid and Related Disorders" highlights the central role of the parathyroids in calcium and skeletal homeostasis by reviewing the molecular genetics of: hyperparathyroidism; hypoparathyrodism; pseudohypoparathyroidism and the McCune–Albright syndrome; disorders of the PTH receptor; disorders of the calcium-sensing receptor; multiple endocrine neoplasia type 1 (MEN1) and type 2 (MEN2). The fifth section "Vitamin D and Renal Disorders" details the advances in the molecular and cellular advances in: renal phosphate wasting disorders; vitamin D dependent rickets type I and II, and the vitamin D receptor; the Fanconi syndromes, Dent's disease and Bartter's syndrome; magnesium disorders; and hypercalciuria. It is our hope that this book and chapters will contribute to a better understanding of the complexity at the genetic, cellular and physiological levels of bone biology and skeletal disorders, and thereby to improvements in diagnosis and treatments for patients.

The task of assembling such a large number of international experts, who agreed to work together and complete

this ambitious venture, was daunting and formidable. However, friendship and good humor prevailed, and the book has been completed in a timely manner and still remains fresh. We are indebted to all authors for delivering their chapters on time, and for remaining friends.

Finally, such an immense undertaking succeeds only with the help of a dedicated and committed team. In particular, we are grateful to Mara Conner, at Elsevier, who encouraged us in this venture and who has worked tirelessly with us to bring this exciting book to you. In addition, we thank Megan Wickline, who with Mara, has helped us to keep on schedule and on course. We hope you will find this book enjoyable and educational; we certainly have!

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SECTION 1

GENERAL BACKGROUND TO GENETICS

CHAPTER

1

Introduction to Genetics of Skeletal and Mineral Metabolic Diseases

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1 INTRODUCTION

Many skeletal and mineral metabolic diseases have a genetic basis, which may be due to a germline single gene abnormality (i.e., a monogenic or Mendelian disorder), a somatic single gene defect (i.e., a postzygotic mosaic disorder) or involve several genetic variants (i.e., oligogenic or polygenic disorder).[1–4] Genetic mutations causing Mendelian diseases usually have a large effect (i.e., penetrance), whereas oligogenic or polygenic disorders are associated with several genetic variations, each of which may have smaller effects with greater or smaller contributions from environmental factors (i.e., multifactorial disorders).[1–4] While the majority of monogenic disorders result from rare mutations affecting the coding sequence of the responsible gene, the majority of common genetic variants identified in association with polygenic traits are located in noncoding regions, typically in proximity to candidate genes implicated in the respective phenotype. Notably, considerable overlap exists between the genes responsible for monogenic skeletal disorders and those contributing to polygenic bone phenotypes. Identification of many of these loci have provided key insights into the molecular pathogenesis of skeletal disease, and provided targets for the development of novel therapeutic agents.[2,3,5] In this chapter, the genetics of bone and mineral metabolic disease, the clinical approach to those that are heritable, and use of the different available genetic tests, are reviewed.

2 GENETICS OF SKELETAL AND MINERAL METABOLIC DISEASES

2.1 Modes of Inheritance

The genetic architecture of skeletal and mineral disease is broadly divided into single-gene monogenic disorders and polygenic complex traits.

Inheritance of monogenic skeletal and mineral metabolic disorders may occur as one of six broad traits (Fig. 1.1, Table 1.1): autosomal dominant [e.g., osteogenesis imperfecta (OI) due to type-I collagen alpha-1 and alpha-2 chain (*COL1A1* and *COL1A2*) mutations, achondroplasia due to fibroblast growth factor receptor 3 (*FGFR3*) mutations]; autosomal recessive (e.g., vitamin D-dependent rickets types I and II due to mutations of the renal 1 alpha hydroxylase and vitamin D receptor genes, respectively); X-linked recessive (e.g., Dent disease due to mutations of a chloride/proton antiporter referred to as CLC-5); X-linked dominant [e.g., X-linked hypophosphatemic rickets due to mutations of the phosphate endopeptidase on the X chromosome (*PHEX*) gene]; Y-linked [e.g., Swyer syndrome (46XY complete gonadal dysgenesis) due to mutations of the sex determining region Y (*SRY*) gene, and azoopermia and oligospermia due to deletions of regions of the Y-chromosome]; and non-Mendelian mitochondrial disorders [e.g., forms of hypoparathyroidism associated with the Kearns–Sayre syndrome and mitochondrial encephalopathy, lactic

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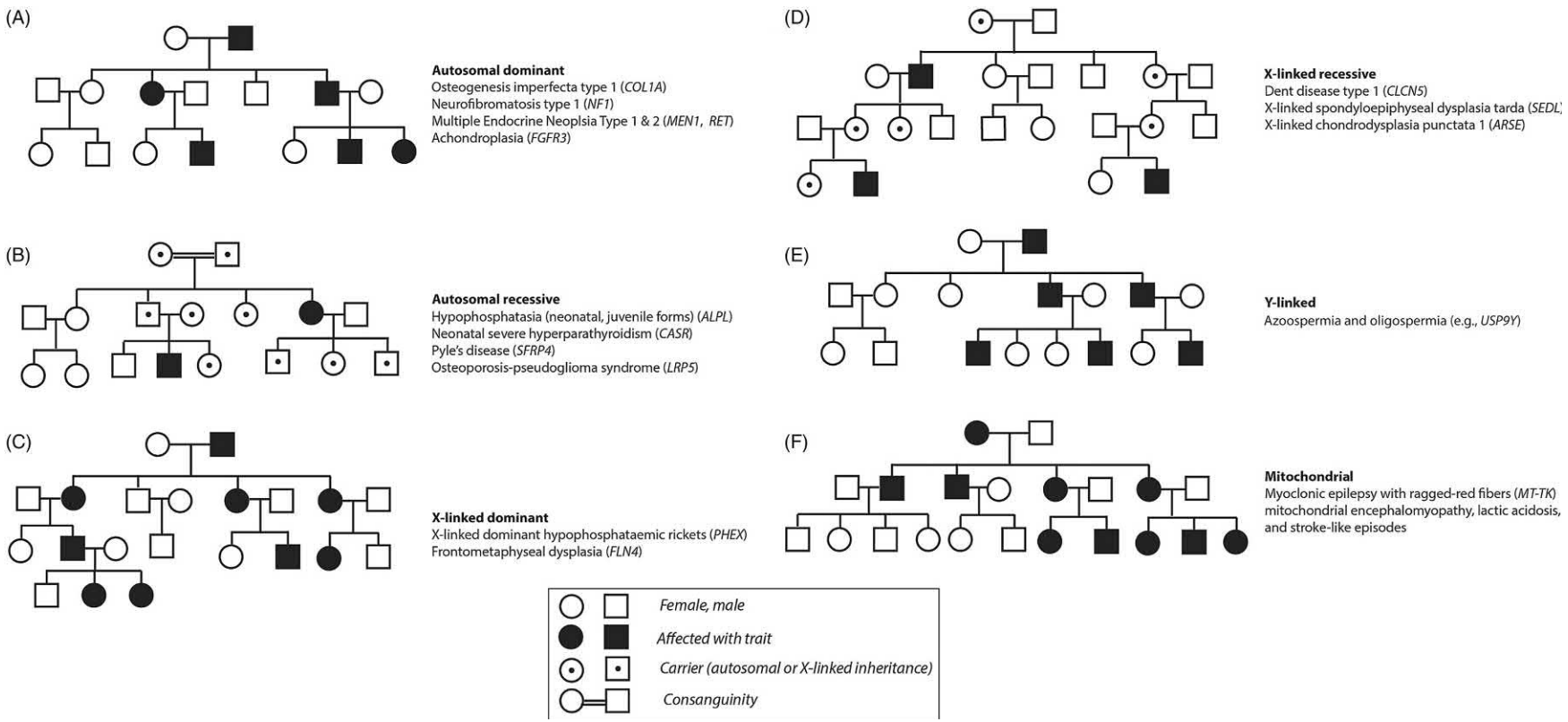

FIGURE 1.1 Modes of inheritance of monogenic skeletal disease. Inheritance of monogenic mineral metabolism and skeletal disorders may occur as one of six main traits: (A) autosomal dominant; (B) autosomal recessive; (C) X-linked dominant; (D) X-linked recessive; (E) Y-linked; and (F) non-Mendelian mitochondrial disorders. Examples of conditions associated with each mode of inheritance are provided with the main causative gene provided in parentheses.

TABLE 1.1 Examples of Monogenic Skeletal Disorders, Modes of Inheritance and Genetic Etiology

Mode of inheritance/disease	Gene(s)	Estimated prevalence	Diagnostic genetic test	Notes
Autosomal dominant				
Osteogenesis imperfecta (OI)	*COL1A1*[a,b]	6–7/100,000	Single gene/gene panel	*COL1A* and *COLA2* responsible for >90% of OI
	COL1A2		Single gene/gene panel	
Stickler syndrome type 1	*COL2A1*	1:10,000	Single gene/gene panel	Represents 80%–90% of cases of Skickler syndrome
Stickler syndrome type 2	*COL11A1*	See above	Single gene/gene panel	Represents 10%–20% of cases of Skickler syndrome. Other rare forms (type 3 and 4) also exist due to mutations in other genes
Hypophosphatemic rickets, autosomal dominant	*FGF23*	Rare	Single gene/gene panel	
Achondroplasia	*FGFR3*	1:15–40,000	Single gene/gene panel	~80% of individuals have a de novo mutation
Multiple epiphyseal dysplasia (MED)	*COMP* (genetic heterogeneity)	1:10,000	Gene panel	Mutations also reported in *COL9A1*, *COL9A2*, *COL9A3*, *MATN3* associated with dominant MED
Neurofibromatosis type 1 (NF1)	*NF1*	1:3,000	Single gene/gene panel	Large gene size makes evaluation by Sanger sequencing costly and time consuming
"High bone mass" (worth-type endosteal hyperostosis)	*LRP5*	Rare	Single gene/gene panel	
Early-onset osteoporosis/juvenile primary osteoporosis	*WNT1/LRP5*	Uncertain	Single gene/gene panel	
Familial hypocalciuric hypercalcemia type 1	*CASR*	1:15–30,000	Single gene/gene panel	Associated with loss of function mutation in *CASR*. Gain of function mutations associated with autosomal dominant hypocalcemia type 1 (ADH1)
Familial hypocalciuric hypercalcemia type 2	*GNA11*	Rare	Single gene/gene panel	Associated with loss-of-function mutations in *GNA11*. Gain of function mutations are associated with autosomal dominant hypocalcemia type 2 (ADH2)
Familial hypocalciuric hypercalcemia type 3	*AP2S1*	Rare	Single gene/gene panel	Associated with loss-of-function mutations in *AP2S1* at arginine residue at codon 15
Familial expansile osteolysis	*TNFRSF11A*	Rare	Deletion/duplication analysis	Associated with duplication within the *TNFRSF11A* gene, which encodes RANK protein
Hypophosphatasia	*TNSALP/ALPL*	Uncertain	Single gene/gene panel	Can also be inherited as autosomal recessive trait
Autosomal recessive				
Osteogenesis imperfecta (OI) Types VII	*CRTAP*	Rare	Single gene/gene panel	Associated with severe clinical features although milder variants may occur

(*Continued*)

TABLE 1.1 Examples of Monogenic Skeletal Disorders, Modes of Inheritance and Genetic Etiology (*cont.*)

Mode of inheritance/disease	Gene(s)	Estimated prevalence	Diagnostic genetic test	Notes
Osteogenesis imperfecta (OI) Type VIII	*P3H1*	Rare	Single gene/gene panel	Associated with severe clinical features including slow growth and early fracture
Osteogenesis imperfecta (OI)Type XV	*WNT1*	Rare	Single gene/gene panel	
Hypophosphatasia	*TNSALP/ALPL*	1:100,000	Single gene/gene panel	Milder forms may occur with autosomal recessive and dominant inheritance patterns
Neonatal severe primary hyperparathyroidism (NSHPT)	*CASR*	Rare	Single gene/gene panel	Typically presents in first 6 months of life often with life-threatening hypercalcemia
Vitamin D-dependent rickets, type 1	*CYP27B*	<1:200,000	Single gene/gene panel	
Vitamin D-dependent rickets, type 2	*VDR*	<1:200,000	Single gene/gene panel	
Multiple epiphyseal dysplasia (MED)	*SLC26A2*	Rare	Single gene/gene panel	
Osteoporosis-pseudoglioma syndrome	*LRP5*	1:2,000,000	Single gene/gene panel	Impaired vision present at birth or during early infancy due to retinal disease
Sclerosteosis type 1	*SOST*	Rare	Single gene/gene panel	More severe than van Buchem disease, occurring in Afrikaner population
Sclerosteosis type 2	*LRP4*	Rare	single gene/gene panel	Can also demonstrate autosomal dominant inheritance
van Buchem disease	*SOST*	Rare	Single gene/gene panel	Identified in individuals of Dutch ancestry. Disease associated with a 52 kb deletion of a regulatory region neighboring the *SOST* gene
Pyle's disease	*SFRP4*	Rare	Single gene/gene panel	Characterized by expanded trabecular metaphyses and increased fracture risk
Juvenile Paget disease	*TNFRSF11B*	Rare	Single gene/gene panel	*TNFRSF11B* encodes the protein OPG which binds RANKL
X-linked dominant				
Hypophosphatemic rickets, X-linked	*PHEX*	1:20,000	Single gene/gene panel	
X-linked recessive				
Dent disease type 1	*CLCN5*	Rare	Single gene/gene panel	~250 families reported in literature
Spondylo-epiphyseal dysplasia tarda, X-linked	*TRAPPC2 (SEDL)*	1:150,000	Single gene/gene panel	
Y-linked				
Azoospermia and oligospermia	Azoospermia factor regions	1:2–3,000	Y-chromosome deletion analysis	Typically results from deletion of multiple genes in "azoospermia factor regions." Deletions of all or part of a single gene *USP9Y* observed in some cases
Swyer syndrome (46, XY gonadal dysgenesis)	*SRY*	1:80,000	Gene panel/FISH/array analysis	15% of cases of Swyer syndrome are due to mutation of the *SRY* gene. Female patients (carrying a male karyotype) may develop osteoporosis because of gonadal dysgenesis

TABLE 1.1 Examples of Monogenic Skeletal Disorders, Modes of Inheritance and Genetic Etiology (*cont.*)

Mode of inheritance/disease	Gene(s)	Estimated prevalence	Diagnostic genetic test	Notes
Mitochondrial				
Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS)	*MT-TL1*	<1:4,000	Single gene/mitochondrial gene panel	Maternal inheritance pattern. m.32423A > G mutation observed in ~80% of patients. Short stature observed in ~50% of patients
Kearns–Sayre syndrome	—	1–3:100,000	Mitochondrial deletion/duplication analysis	Typically presents with progressive external ophthalmoplegia. Most common genetic abnormality involves ~5 kb DNA deletion including 12 mitochondrial genes
Mosaicism				
McCune–Albright syndrome (polyostotic fibrous dysplasia)	*GNAS*	1:100,000–1,000,000	Single gene/gene panel	Postzygotic somatic mosaicism
OI[b]	*COL1A1/COL1A2*	See above	Single gene/gene panel	Germline mosaicism may mimic autosomal recessive inheritance pattern
Neurofibromatosis type 1	*NF1*	Rare	Single gene/gene panel	Postzygotic somatic mosaicism may give rise to "segmental" NF1 with disease limited to region of the body
Melorheostosis	Gene uncertain	~1:1,000,000	Not applicable	Presumed postzygotic mosaicism perhaps involving KRAS
Proteus syndrome	*AKT1*	Rare	Single gene/gene panel	Postzygotic somatic mosaicism

MED, Multiple epiphyseal dysplasia; NF1, neurofibromatosis type 1, NSHPT, neonatal severe primary hyperparathyroidism; OI, osteogenesis imperfecta.
Different conditions may be due to mutations in the same gene. For example, mutations in *COL1A1* are most commonly associated with OI (although may manifest different clinical features), but may also result in Caffey disease (characterized by infantile cortical hyperostosis) and arthrochalasia type Ehlers–Danlos syndrome.
a Apparently similar condition may be due to mutations in different genes and may display different patterns of inheritance. For example, OI and MED may occur as autosomal dominant or autosomal recessive disease due to mutations in several different genes.
b Some autosomal disorders may also arise in the context of mosaicism, either from postzygotic somatic mosaicism in the developing fetus, or from germline mosaicism in an apparently unaffected parent.

acidosis, and stroke (MELAS) syndrome].[6] Inheritance of underlying polygenic disorders may be revealed by a familial occurrence but the pattern of transmission may be complex because of environmental factors. Examples of polygenic skeletal and mineral disorders include osteoporosis, osteoarthritis, and hypercalciuria. In addition, it is important to note that polygenic traits, such as osteoporosis, may rarely occur in monogenic forms, and in this setting can be easily overlooked, for example, X-linked osteoporosis due to mutations of the Plastin 3 (*PLS3*) gene, or early-onset osteoporosis due to heterozygous mutations of the Wnt family member 1 (*WNT1*) gene.[7–9] In addition to the "classical" Mendelian modes of inheritance, occasional kindreds exhibit apparent inherited disease but due to alternate mechanisms. For example, germline mosaicism (in which a postzygotic mutation occurs during or prior to gametogenesis in a parent) may give rise to a seemingly autosomal recessive pattern of inheritance with multiple affected offspring of apparently unaffected parents.[10] Finally, disease traits may be conditioned by epigenetic mechanisms (e.g., methylation), although the current understanding of such processes remains limited. In fact, combinations of genetic and epigenetic changes occasionally influence disease expressivity, which may include parent-of-origin effects. For example, germline mutations plus epigenetic changes at the guanine nucleotide-binding protein alpha subunit gene (*GNAS*; protein GSα) locus are responsible for a spectrum of pseudohypoparathyroidism phenotypes; thus, maternally inherited inactivating coding region mutations in *GNAS* give rise to pseudohypoparathyroidism type 1A (PHP1A), while the equivalent paternally inherited mutations give rise to pseudopseudohypoparathyroidism. However, a related phenotype of pseudohypoparathyroidism type 1B (PHP1B), which occurs in both familial and sporadic forms, is observed

in association with the loss-of-methylation of regulatory regions at the maternal *GNAS* locus, which results in reduced Gsα expression and parathyroid hormone resistance.[11]

While polygenic traits, such as bone mineral density (BMD) and to a lesser extent fracture risk, are highly heritable, the majority of "heritability" remains unexplained. On an individual patient basis in the clinical setting, assessing the genetic contribution to such polygenic traits remains challenging, although it is important to recognize that polygenic traits may rarely occur as part of either isolated or wider syndromic monogenic disorders.

2.2 Genetic Heterogeneity and Monogenic Skeletal Diseases

The appropriate evaluation, investigation, and treatment of patients with skeletal and mineral metabolic disorders requires familiarity with the great diversity of phenotypes which may occur due to underlying hereditary disease. For example, a recent classification indicates that there are >400 specific genetic skeletal disorders.[1] Thus, arriving at the correct diagnosis will depend on a combination of careful clinical work-up, appropriate laboratory investigation and genetic testing, and an awareness of the potential pitfalls that may be present along the way.[1] While the diagnosis of some monogenic disorders may be apparent from pathognomonic or characteristic clinical or radiographic features, many disorders share overlapping or phenotypically indistinguishable findings (e.g., reduced bone mass, skeletal fragility, abnormal mineralization). Similarly, while certain disorders result from mutation(s) in a single culpable gene, other diseases represent marked genetic heterogeneity with mutation(s) in one of many candidate genes. Although establishing the likely mode of inheritance together with careful clinical and routine laboratory evaluation may provide further diagnostic clues, in some situations a broad genetic differential persists. For example, although most cases of OI are due to mutations in the type-I collagen genes (i.e., *COL1A1*, *COL1A2*) many additional genes (~10–15), which are typically involved in posttranslational processing of collagen, account for a small percentage of OI cases.[5,7,12,13] Similarly, heritable disorders characterized by impaired bone mineralization including hypophosphatemic rickets have "genetic heterogeneity"; that is, several genetic etiologies, as do many other conditions including osteopetrosis and Ehlers–Danlos syndrome.[14]

In contrast to the situation in which phenotypically similar disorders may be caused by mutations in one of many different genes (e.g., osteopetroses), sometimes mutations in the same gene underlie a spectrum of clinical phenotypes with some considered distinctive diseases (e.g., disorders from constitutive activation of receptor activator of NF-κB, RANK). In some instances, disease severity is determined by whether a mutation is carried in the heterozygous or homozygous state, or the type or location of the mutation within the gene (i.e., loss-of-function, missense amino acid change). For example, the severity of hypophosphatasia, the inborn-error-of-metabolism featuring low serum alkaline phosphatase activity, reflects the number and nature of the mutation(s) involving the *TNSALP* (*ALPL*) gene that encodes the tissue-nonspecific isoenzyme of alkaline phosphatase (Chapter 28). The severe perinatal and infantile forms of hypophosphatasia are inherited in an autosomal recessive manner, while the later-onset and more mild forms are often inherited in an autosomal dominant fashion.[15] In contrast, single heterozygous mutations in genes, such as *FGFR3*, collagen type-II alpha-1 (*COL2A1*) and collagen type XI, alpha-1 (*COL11A1*) and alpha-2 (*COL11A2*) each give rise to what have been considered separate or unique disorders despite similar patterns of inheritance. For example, for different individual mutations in *FGFR3*; missense mutations resulting in the amino acid change Gly380Arg cause most cases of achondroplasia; mutations resulting in Pro250Arg and Lys650Met cause Muenke syndrome (characterized by craniosynostosis) and severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN), respectively; while a number of different *FGFR3* missense mutations are reported in patients with hypochondroplasia and type 1 thanatophoric dysplasia.[1] Given this apparent genetic/phenotypic complexity despite genetic "homogeneity," establishing the genetic diagnosis may be challenging, yet crucial for the evaluation of patients and kindreds in establishing the likely mode of inheritance.

2.3 Identifying Genetic Abnormalities Causing Monogenic Diseases

Traditionally, the identification of causative genes for Mendelian monogenic diseases involved characterization of the involved genes and then determining the DNA sequence alterations (i.e., mutations) either after: (1) cytogenetic analysis to detect chromosomal abnormalities, and/or (2) after studying affected kindreds for cosegregation with genetic markers (polymorphisms) to define the chromosomal location.[2,5] These "classical" gene-discovery approaches are now increasingly replaced by next-generation DNA sequencing of affected kindreds or patients (i.e., whole-exome or whole-genome approaches).[16,17] Together, these methods have defined the genetic basis of many monogenic skeletal disorders, which typically result from mutations affecting the coding sequence of their causative genes, which in turn compromise the gene's function.

2.4 Identifying Genes Causing Polygenic Traits

In contrast to Mendelian disorders, the genetic investigation of common polygenic traits (e.g., osteoporosis, osteoarthritis) have utilized increasingly large populations of cases and controls (or the extreme spectra of phenotype), which facilitate genome-wide association studies (GWAS).[2–5] Such studies typically involve direct or imputed genotyping of large numbers of common (e.g., minor allele frequency >5%) and infrequent (e.g., minor allele frequency 1%–5%) single nucleotide polymorphisms/variants (SNPs/SNVs) to identify genetic loci enriched for the trait. Usually, each variant is associated with modest effect sizes and is often under strong environmental influence. Most frequently, these loci are outside of gene coding regions, and therefore ascribing functionality may be challenging.[2–5] Candidate genes contributing to the trait are commonly selected from their proximity to the identified variant; for example, >100 candidate genes have been identified from GWAS studies investigating osteoporosis, although it is important to note that these large number of loci only account for a small percentage of the heritability of the trait.[2–5] This indicates that either many more additional common genetic variants with small effect sizes remain to be identified, or that rare variants with larger effect sizes may contribute. Alternatively, it is also possible that other mechanisms, such as epigenetic regulation (e.g., methylation status of specific genes) contribute to the heritability of specific polygenic traits, although investigating these mechanisms remains challenging (e.g., due to limited availability of relevant tissue for evaluation). For example, a recent genome-wide methylation study using cartilage tissue from a small number of patients with osteoarthritis, identified a number of differentially methylated genomic regions including several implicated in skeletal system morphogenesis and development.[18]

2.5 Molecular Insights From the Investigation of Monogenic Disorders and Polygenic Traits

In the clinical setting, the main value of genetic testing resides in the identification of information that may benefit the patient and wider family (Table 1.2). However, one of the major rewards of the genetic investigation of monogenic disease and polygenic traits has been the fundamental insights that they have provided into the molecular regulation of skeletal health and mineral homeostasis. In particular, these approaches have delineated roles for genes regulating key skeletal processes, which include: signaling pathways involved in bone development; maintenance of skeletal microarchitecture (i.e., through bone modeling and remodeling); and transmission of endocrine and paracrine signals. Indeed, bone and mineral health is orchestrated by a highly complex interplay of elements, which includes: matrix proteins; osteoclast and osteoblast function; hormones (e.g., parathyroid hormone, 1,25-dihydroxycholecalciferol); and developmental and regulatory transcription factors and signaling pathways.[2,5] Roles for several of these components have primarily been established through genetic investigation including the Wnt and osteoprotegrin (OPG)—receptor activator of NF-κB ligand (RANKL) signaling cascades.[2,5] For example, mutations in several Wnt pathway components are associated with monogenic disorders: autosomal-recessive loss-of-function mutations of the low-density lipoprotein receptor-related

TABLE 1.2 Value of Genetic Testing in Clinical Practice

Value of genetic testing in patients with skeletal and mineral metabolic diseases
Benefits for the patient
• Appropriate investigation and treatment of disease • Screening/surveillance for associated features not clinically apparent • Prognostic information regarding disease course
Benefits for first-degree relatives and/or progeny
• Identification of first-degree family members at risk of disease or those at risk of passing on to their progeny • Identification of family members who do not harbor the genetic abnormality thereby alleviating the anxiety and burden of disease from them and/or their progeny • Where appropriate, to enable preconception genetic counseling • Where appropriate, to enable prenatal diagnosis
Academic/research benefits
• Improved molecular characterization and understanding of associated disorder • Development of novel therapeutic targets/pathways
Potential future clinical benefits
• Advent of personalized medicine—matching therapies to genetic defects

protein 5 (*LRP5*) gene that encodes a key Wnt coreceptor, result in osteoporosis-pseudoglioma syndrome, while heterozygous activating mutations in *LRP5* result in autosomal dominant high bone mass.[2,5,19] Additionally, individuals with autosomal recessive loss-of-function mutations of the Wnt-β-catenin regulator sclerostin (*SOST*), manifest the sclerosing bone dysplasias sclerosteosis, while patients carrying a biallelic 52 kb deletion of an enhancer element downstream of the *SOST* gene express van Buchem disease.[20,21] Biallelic loss-of-function mutations of *WNT1* causes an autosomal recessive form of OI, while heterozygous carriers of such *WNT1* missense mutations develop autosomal dominant early-onset osteoporosis.[7,22] More recently, biallelic truncating mutations in secreted frizzled-related protein 4 (*SFRP4*), which encodes a soluble Wnt inhibitor, were reported in patients with Pyle's disease, a disorder characterized by cortical-bone thinning, limb deformity, and fracture.[23] Further supporting a key role in bone biology, many Wnt pathway components (>15 genes), including *LRP5* and *SOST*, have been identified as candidate genes from GWAS studies of BMD, and these genetic approaches are defining increasingly specific functions of Wnt components, including the discovery that *WNT16* is reportedly a key determinant of cortical bone strength.[2–5]

A similar overlap of genes implicated in monogenic and polygenic phenotypes is observed for members of the OPG/RANKL/RANK/NF-κB pathway, which is a major regulator of osteoclast differentiation and function. Thus, patients harboring mutations of the tumor necrosis factor receptor superfamily member 11B (*TNFRSF11B*), which encodes OPG, and *TNFRSF11A* that encodes RANK, manifest the monogenic disorders juvenile Paget disease (autosomal recessive) and familial expansile osteolysis and its variant disorders (autosomal dominant), respectively. Furthermore, both these genes (and other components of the OPG/RANKL/RANK/NF-κB pathway) have been identified from GWAS studies to be quantitative trait or loci associated with BMD.[2–5]

In addition to the investigation of common variants in polygenic traits, recent studies have established a role for rare and/or low-frequency variants with larger effect sizes, which provide additional insight into bone biology. For example, specific to the Icelandic population, a rare truncating variant of the leucine-rich receptor containing G-protein-coupled receptor (*LGR4*) gene was reported to be associated with a variety of phenotypic traits including low BMD and osteoporosis,[24] while two rare coding region variants in *COL1A2* were also identified in this population in association with low BMD.[25] A further study of individuals of European ancestry reported a low-frequency noncoding variant in close proximity to the Engrailed homeobox-1 (*EN1*) gene, which was associated with ~four- and ~threefold larger effect size on BMD and fracture risk, respectively, than previously reported common variants.[26] Furthermore, this and other studies support a key role for the *EN1* gene in bone biology, likely through an interaction with Wnt factors, and regulation of bone turnover, thereby highlighting the potential utility of these genetic approaches for investigating the molecular pathogenesis of skeletal and mineral metabolic diseases.[27,28]

2.6 Genetic Understanding and Application to Development of Novel Therapeutics

A principal aim of refined genetic characterization of skeletal disease and disorders or bone mineral homeostasis is the identification of genes and pathways that may be targeted therapeutically. The considerable overlap in genes identified from the investigation of monogenic disease and polygenic traits indicates that these genes and the pathways regulated by them, likely represent good candidates. Indeed, many existing therapies, as well as those in development, target key components identified from these studies. For example, the monoclonal antibody denosumab blocks RANKL within the OPG/RANKL/RANK/NF-κB signaling pathway to inhibit the formation and activity of osteoclasts, thereby inhibiting bone resorption.[5] Similarly, several drugs in development are directed at the Wnt pathway. This includes antisclerostin antibodies (e.g., romosozumab), which are being evaluated as an anabolic treatment for osteoporosis, with the aim of simultaneously increasing bone formation and inhibiting bone resorption through direct and indirect targeting of osteoblast and osteoclast function, respectively. Future drug and biologic development will surely include the targeting of genetically identified pathways, perhaps with greater specificity with the aim of personalized therapies. For example, the multinational approval in 2015 of the bone-targeted enzyme-replacement biologic asfotase-alfa to treat typically pediatric-onset hypophosphatasia, emphasizes the importance of determining the genetic, molecular, and biochemical basis for a skeletal/mineral metabolic disease.[15] Similarly, assessing treatment response according to genetic etiology has been investigated, as illustrated by a recent study, in which patients with early-onset low-turnover osteoporosis due to *WNT1* or *PLS3* mutations, were demonstrated to respond to teriparatide treatment.[29]

3 APPROACH TO THE PATIENT WITH GENETIC SKELETAL/MINERAL METABOLIC DISEASE

3.1 Clinical Approach

Skeletal and mineral metabolic disorders may present with an array of clinical symptoms and signs and

an awareness of the spectrum of potential phenotypes is key to appropriate assessment, further investigation, and treatment.[30–33] The diagnosis and treatment of these hereditary disorders requires clinical skill. Clinicians have at their disposal many biochemical and radiological tools to aid diagnosis, but they should be used judiciously and following the acquisition of a detailed personal and family history and the undertaking of a careful clinical examination.[34–37] The possibility of hereditary disease could tempt the physician into hasty genetic testing, but this should be avoided until as much relevant information has been acquired to ensure the maximum utility of the available tests. As the range and complexity of molecular genetic testing increases, selecting among them may be challenging and many factors need to be considered. In addition, the appropriate interpretation of genetic test results requires the physician to recognize and assess potential uncertainties and limitations. Finally, it is important to fully include the patient in decision-making processes and ensure that informed consent is acquired beforehand.

3.2 Medical History and Physical Examination

Diagnosis and treatment of metabolic and dysplastic skeletal disease, perhaps especially those that are heritable, begins by acquiring information that can come from the patient's stated medical history and the findings from thorough physical examination.[38] The importance of the medical history cannot be overemphasized. It determines if any of the many adverse exogenous factors that complicate dysplastic or metabolic bone disease will be uncovered. A questionnaire may be a beginning, but is hardly a substitute. Only by talking with his/her patient will the physician sense how knowledgeable this individual might be and judge the value of the information. Subsequently, the medical history should be reported as a narrative to capture the clinical problem(s). Paramount is orderly accumulation, documentation, and consideration of the information acquired directly from patients. This effort helps to disclose potentially important medical records, guide the physical examination, and laboratory studies including any genetic testing, and choose safe and effective therapy.

Most genetically based dysplastic and metabolic bone diseases are chronic conditions. The "history of present illness" may be lengthy, but provides infrastructure for diagnosis and therapy. Critical clues concerning etiology and pathogenesis should emerge perhaps with a glimpse at prognosis. Here the physician can learn if previous medical records, radiographs, laboratory tests, etc., can help in diagnosis and prognostication. Have the signs and symptoms been lifelong, or begun recently prompting very different diagnostic considerations and interventions? Has the patient been compliant with medical care; if not, will therapy be safe? With this effort, the physician can also provide the basis for sound clinical research. Physical examination can reveal a considerable variety of findings for diagnosis including skeletal deformities especially common and unique in children, but there are also complications, which may include bone abnormalities that require attention for successful treatment. The diagnosis of such a disorder may emanate from recognition of a single physical finding; for example, blue or gray sclerae (OI), large café-au-lait spots (McCune–Albright syndrome), premature loss of deciduous teeth (hypophosphatasia), hallux valgus (fibrodysplasia ossificans progressiva), alopecia in some patients with hereditary vitamin D-resistant rickets (vitamin D-dependent rickets, type II), brachydactyly (pseudohypoparathyroidism, type IA), or numerous surgical scars [multiple endocrine neoplasia (MEN) syndromes]. For some genetic metabolic bone diseases, a constellation of physical findings suggests the diagnosis; for example, rickets featuring craniotabes at birth, a rachitic rosary (enlargement of the costochondral junctions) appearing during the first year of life. In infancy or childhood, bowed legs, short stature, flared wrists, and ankles reflect metaphyseal widening. There can be Harrison's groove (rib cage ridging from diaphragmatic pull producing a horizontal depression along the lower border of the chest at costal insertions of the diaphragm). Although weight bearing typically bows rachitic lower limbs, knock-knee deformity may instead occur especially if the rachitic disturbance occurs during the adolescent growth spurt. In adults, skeletal deformation perhaps originating in childhood can cause much of the morbidity from metabolic bone disease. Bowing of the lower limbs predisposes to osteoarthritis, especially in the knees. Without a complete physical examination, these important problems may go unnoticed.

3.3 Family Medical History for Determining Mode of Disease Inheritance

Many mineral metabolic and skeletal disorders have a monogenic etiology, and this may be suspected because of an early age of onset, occurrence of other abnormalities consistent with a syndromic disease, or a family history of the disease. The "family history" (or FH) concerning metabolic bone disease is vital for revealing the mode of inheritance of the disorder (Fig. 1.1). For example, consanguinity that is apparent, or inapparent involving geographical location and a "founder" mutation, can be an important clue for autosomal recessive conditions; whereas the autosomal dominant disorders may be disclosed by prior or prospective study of relatives [e.g., familial benign (hypocalciuric) hypercalcemia or OI]. Inborn errors of vitamin D bioactivation or resistance are rare, and the FH may be key to considering

one, because of national or ethnic background. Furthermore, significant information can come from screening studies to identify and treat or counsel affected relatives who may provide important clues to the patient's future complications and prognosis, or represent "carriers." Medical records from similarly affected living or deceased family members may establish the diagnosis, guide prognostication, and indicate a safe and effective treatment. Reporting that the FH is "negative" without first considering the basis for this information might be misleading. If the patient is adopted, he/she is less likely to give useful details. Knowing the size of the family is essential before dismissing possible transmission of a heritable disorder. The only child of only children, or the patient from a disrupted family, is not as likely to disclose a heritable disorder as one from a large cohesive kindred.

A detailed FH will help to reveal a pattern of inheritance that will provide critical clues regarding the mode of Mendelian inheritance (Table 1.1, Fig. 1.1). Thus, in autosomal dominant diseases, the affected person (unless a "new mutation" sporadic case) will have one affected parent and the disease occurs in both sexes and may be transmitted by the father or mother. In autosomal recessive diseases, which affect both sexes, the affected person is usually born to asymptomatic "carriers" who may be related (i.e., consanguineous). In X-linked recessive diseases, only males are usually affected, who are born to often unaffected parents of whom the mother is an asymptomatic carrier with affected male relatives, and there is no male-to-male transmission. In X-linked dominant diseases, both males and females are affected, although the females are typically more mildly and variably affected than males, and 50% of children (girls and boys) from an affected woman will have the disease, and 100% of the daughters, but 0% of sons, of an affected man will have the disease. In Y-linked diseases, only males are affected, and the affected males will have an affected father (patrilineal), and all the sons of an affected man will be affected. Mitochondrial (non-Mendelian) inherited disorders can affect both sexes and are transmitted only by an affected mother in her mitochondrial, not genomic, DNA. This is because the small volume of a sperm precludes it from contributing mitochondria to the zygote, and thus all mitochondrial DNA is inherited from mothers (i.e., matrilineal inheritance). These patterns of inheritance may be complicated by: nonpenetrance or variable expression in autosomal dominant disorders [e.g., MEN type 1 (MEN1)]; imprinting whereby expression of an autosomal dominant disorder depends whether it is maternally or paternally transmitted (e.g., pseudohypoparathyroidism type 1A and pseudopseudohypoparathyroidism); anticipation, whereby some dominant disorders become more severe (or have earlier onset) in successive generations; pseudodominant inheritance involving autosomal recessive disorders reappearing in successive generations within small-/medium-sized populations; and mosaicism in which an individual, who has developed from a single fertilized egg, has two or more populations of cells with different genotypes because of postzygotic mutations (e.g., McCune–Albright syndrome). However, germline mosaicism within eggs or sperm, arising from somatic mutation during gametogenesis, may lead to diagnostic and recurrence risk confusion, because seemingly unaffected parents may have multiple affected offspring, suggesting autosomal recessive inheritance but actually reflecting an autosomal dominant disorder (e.g., OI II). Similarly, separating individuals with apparent polygenic disease traits from those with monogenic disease may be difficult, and may only be revealed by the acquisition of a full family history. In summary, these patterns of inheritance, which can help to establish a genetic disorder in a family and identify additional individuals at risk of disease, may only be elucidated by taking a detailed family history.

4 CURRENT GENETIC TESTS, THEIR CLINICAL UTILITY, AND INTERPRETATION

4.1 Clinical Value of Genetic Testing

The identification and delineation of a genetic basis for a patient's disorder has many potential benefits (Table 1.2). They include the appropriate investigation and treatment of the disorder, as well as evaluating the patient for any associated features that may not be clinically apparent at initial evaluation. For some individuals, a genetic diagnosis may offer prognostic information and also facilitate appropriate genetic counseling, as well as the testing of relatives who may be asymptomatic "carriers." Furthermore, family members found not at risk of the disease (or of passing the disease to their children) can be reassured. For parents with children affected with significant skeletal disease, the identification of a genetic diagnosis may facilitate appropriate preconception genetic counseling and/or prenatal genetic testing in future pregnancies. As emphasized earlier, genetic testing may also directly or indirectly lead to an improved understanding of disease pathogenesis, which in turn may ultimately provide avenues for novel drug discovery and personalized treatment approaches.[3–5]

4.2 Pretest Considerations—Which Test?

Several factors must be considered before requesting genetic testing. These include: the phenotype of the patient in question, likely mode of inheritance, availability of additional pedigree members to aid the diagnosis, and

potential type(s) of genetic abnormality present. Indeed, the ability to undertake sequencing of "trios" (i.e., both parents and the affected offspring) may allow the identification of autosomal recessive, compound heterozygous, or de novo mutations in the patient, which might not be possible in the absence of parental samples.[39]

Many types of genetic abnormality [e.g., single gene defect, copy number variation (CNV), or aneuploidy] may result in skeletal or mineral metabolism disease. Thus, most monogenic disorders typically involve either SNVs or small insertions or deletions (indels) affecting the coding region of a given gene. For example, disease-associated SNVs typically result in nonsynonymous amino acid substitutions, nonsense mutations (i.e., resulting in the introduction of a stop codon), or splicing defects (i.e., due to disruption of donor or acceptor splice sites at the intron/exon boundary); in contrast, indels frequently disrupt protein function through either in-frame or out-of-frame insertions or deletions leading to either the insertion or loss of one or more amino acids, or an alteration in both the amino acid sequence and stop codon location, respectively. The detection of the majority of these monogenic defects will typically require high-resolution DNA sequencing for their identification. However, whole or partial gene deletions (see later) may also account for a variable percentage of patients with monogenic syndromes, which may go undetected by direct sequencing methods. In contrast to small genetic changes responsible for the majority of monogenic disease (i.e., SNVs, indels) some disorders are characterized by marked chromosomal abnormalities. This may include an alteration in the correct number of chromosomes (e.g., aneuploidy in Turner's syndrome), CNVs, which represent large gains or losses of genetic material (e.g., deletion on chromosome 15 in Prada–Willi syndrome), or other structural variants including translocations or inversions. The detection of these large-scale genomic abnormalities has typically involved approaches (e.g., karyotyping) other than direct DNA sequencing, although these are now increasingly identified by next-generation sequencing (NGS) methods [e.g., whole genome sequencing (WGS)].

When undertaking genetic testing it is important to remember that a negative result does not necessarily exclude the presence of genetic disease but rather may reflect: an alternative genetic etiology to the one being tested; limitations of the genetic methodology employed (i.e., inadequate resolution or coverage); or incorrect assumptions regarding the clinical phenotype or mode of inheritance. Details of some of the available tests are provided in Table 1.3. The most appropriate genetic approach is likely to be governed by the estimated pretest probability of identifying a defect in a given gene. For example, where the clinical presentation points to a likely causative gene, single gene sequencing may be appropriate, whereas, when the potential genetic etiology is broad or unknown, the sequencing of multiple genes or genome-wide approaches may be required. Additional considerations may include cost, availability of NGS platforms, and access to bioinformatic expertise.

Currently, genetic testing for those with apparent solitary polygenic traits (e.g., reduced BMD) is rarely indicated, although it may occasionally be used if specific features in the history, examination, or investigation (i.e., early age of onset, family history, atypical clinical features), indicate a significant genetic component. In the future, genetic testing based on genotyping of large numbers of risk alleles may be developed to stratify future risk of bone disease (i.e., fracture risk), although to date the evaluation of such tools has not proved clinically useful, and this likely reflects the relatively modest amount of heritability such loci currently explain, and the limited proportion of variants associated with BMD that are also associated with osteoporotic fracture.[40,41]

4.3 Detection of Chromosomal Abnormalities, Copy Number Variations, and Mutations Causing Disease

An increasing number of molecular genetic tests are available to the clinician, and it is important to note that many of the tests can have overlapping utility. Thus, selecting the most appropriate test(s) requires understanding of both the resolution of the test and its potential limitations. A brief description of the main molecular genetic tests currently available is provided below.

4.3.1 Karyotype

Conventional karyotyping is frequently the first test to evaluate for major chromosomal abnormalities including aneuploidy (i.e., abnormal number of chromosomes), insertions, deletions, duplications, inversions, or reciprocal translocations.[42,43] Such abnormalities may be suspected in the presence of major congenital abnormalities, marked developmental delay, or features of a specific chromosomal abnormality disorder. Typically, karyotyping is performed using peripheral blood leucocytes (isolated from a heparinized blood sample), which are initially cultured, prior to evaluation by high-resolution G-banding (Giemsa staining) of at least 20 metaphase nuclei. The evaluation of multiple cells not only allows a reliable assessment of each chromosome, but also facilitates the identification of mosaicism.[42,43] Occasionally, evaluation of tissues other than blood is required to identify forms of mosaicism (e.g., fibroblasts). Although high-resolution G-band karyotype analysis will identify the majority of major chromosomal defects, its resolution is limited to ~5–10 Mb of DNA, and will therefore not identify smaller abnormalities (e.g., CNVs).[42,43]

TABLE 1.3 Examples of Genetic Tests, Their Molecular Resolution, and Utility

Genetic test	Resolution	Abnormalities detected	Additional notes
Detection of chromosomal abnormalities including CNVs			
Karyotype: G-banding (trypsin-giemsa staining)	5–10 Mb	Aneuploidy Large chromosomal deletions, duplications, translocations, inversions, insertions	Limited resolution Requirement to study many cells to detect mosaicism
Fluorescence in situ hybridization (FISH)	50 kb–2 Mb (dependent on size of probes employed)	Structural chromosomal abnormalities (e.g., microdeletions, translocations)	Labor-intensive Low resolution limits its use Unsuitable where unknown genetic etiology
Multiplex-ligation probe amplification (MLPA)	Probe dependent 50–70 nucleotides Single exon deletion or duplication possible	CNVs including (partial) gene deletions or duplications)	Low cost, technically simple method Simultaneous evaluation of multiple genomic regions Not suitable for genome-wide approaches Not suitable for analysis of single cells
Array comparative genomic hybridization (aCGH)	10 kb (high resolution) 1 Mb (low resolution) (dependent on probes set)	Genome-wide CNVs	Inability to detect balanced translocations Useful for detection of low level mosaicism
Single nucleotide polymorphism (SNP) array	~50–400 kb (dependent on probe set)	Genome-wide detection of SNP genotypes CNVs	Inability to detect balanced translocation Useful for detection of low level mosaicism Detection of copy number neutral regions of absence of heterozygosity (i.e., due to uniparental disomy)
Detection of monogenic disorders (and *CNVs*)			
First-generation sequencing (Sanger)			
Single gene test	Single nucleotide (exonic regions and intron/exon boundaries of candidate gene)	SNVs Small insertions or deletions (indels)	Relative high cost/base May miss large deletions/duplications Unsuitable where unknown genetic etiology
Next-generation sequencing			
Disease-targeted gene panels	Single nucleotide (exonic regions and intron/exon boundaries of candidate genes)	SNVs Small insertions or deletions (indels)	May lack complete coverage of exomic regions (may require Sanger sequencing to fill in "gaps") Increased likelihood of identifying variants of uncertain significance (VUS) as number of genes increases Unsuitable where unknown genetic etiology
Whole exome sequencing (WES)	Single nucleotide (all exonic regions and intron/exon boundaries)	SNVs Small insertions or deletions (indels) CNVs	Not all exons may be covered/captured Difficulties with GC-rich regions and presence of homologous regions/pseudogenes Small indels may not be captured Bioinformatic expertise required for data analysis High likelihood of IFs and VUSs Detection of CNVs requires additional data analysis (i.e., LOH mapping across exonic regions) Suitable for disease-associated gene discovery
Whole genome sequencing (WGS)	Single nucleotide	SNVs Small insertions or deletions (indels) CNVs (Translocations/ rearrangements)	Relative high cost Large data sets generated and complex data analysis requiring bioinformatic expertise Very high likelihood of IFs and VUSs CNV analysis possible but may present specific challenges Suitable for disease-associated gene discovery

CNVs, Copy number variations; FISH, fluorescence in situ hybridization; IFs, incidental findings; LOH, loss of heterozygosity; WES, whole exome sequencing; WGS, whole genome sequencing.

4.3.2 Fluorescence In Situ Hybridization (FISH)

The detection of chromosomal abnormalities and genomic imbalances not detected by a conventional karyotype requires alternate methods. Fluorescence in situ hybridization (FISH) is a technique that employs molecular DNA probes, which are designed to hybridize to specific target regions on metaphase chromosomes, enabling visualization under a fluorescence microscope.[42,43] A variety of chromosomal abnormalities may thus be identified resulting from: the absence of probe binding (e.g., due to a deletion), additional probe binding (e.g., due to a duplication), or probes binding to an aberrant chromosomal region (e.g., due to a translocation or inversion). The molecular resolution of FISH depends on the size of probes used but is typically in the region of 50 kb–2 Mb. The major limitation is that it does not allow genome-wide analysis, but rather is very narrowly limited to detecting abnormalities covered by the probe sets. Alternate FISH-based methods that allow the simultaneous evaluation of several regions of interest have been developed including use of whole-chromosome painting probes [termed multiplex FISH (M-FISH) and spectral karyotyping], with each chromosome labeled a different color.[42,43] While such techniques allow the identification of interchromosomal abnormalities including translocations, they will not identify small deletions, duplications, or inversions.[42,43]

4.3.3 Multiplex-Ligation Dependent Probe Amplification (MLPA)

Multiplex-ligation dependent probe amplification (MLPA) is a method that employs a pool of custom-designed probes to specific genomic regions of interest, and is used to detect specific small chromosomal abnormalities (i.e., single or partial gene deletions).[42] Adjacent probes hybridize to each other and undergo ligation followed by polymerase chain reaction (PCR) amplification of the ligated product, with products separated by electrophoresis. Detection of abnormalities is dependent on the probes employed, which are typically designed to identify specific small gene deletions (e.g., *MEN1* partial or complete deletion). In addition, MLPA may be used in specific circumstances to evaluate for alterations in methylation [e.g., pseudohypoparathyrodism 1b (PHP1b) in which deletion of one or more of four differentially methylated regions is typically observed].[11]

4.3.4 Whole Genome Arrays

The use of whole genome arrays negates the requirement for prior knowledge of the likely chromosomal abnormality responsible for the clinical phenotype.[42,44] Their increased resolution has transformed the field of molecular cytogenetics; although this resolution presents several challenges, not least the recognition that all healthy individuals harbor small CNVs (e.g., 5–10) without any discernable impact on health, while several apparently pathogenic CNVs do not result in disease in all individuals because of reduced penetrance.

4.3.5 Microarray-Comparative Genomic Hybridization (aCGH)

The use of microarray-comparative genomic hybridization (aCGH) for clinical "molecular karyotyping" is increasingly popular and is often employed as a first-line test to detect small chromosomal abnormalities (i.e., CNVs), particularly in young children with early developmental delay, congenital abnormalities, abnormal growth, or additional features, such as autism or epilepsy. The technique of aCGH is based on comparing the DNA from the patient (i.e., from an EDTA blood sample) with that of a control sample. In practice, the patient's DNA is fragmented and compared with a set of immobilized control DNA sequences, which have been deposited on the array platform.[42–44] The resolution of the test will depend on the number and distance between the DNA clones selected on the array. The analysis is performed by mixing the patient's sample (DNA labeled green) with a reference sample (DNA labeled red), prior to applying the samples to the array platform for competitive hybridization with the immobilized reference DNA fragments. Automated analysis of the array measuring red–green fluorescence enables the identification of deletions (appearing as an excess of red) and duplications (appearing as an excess of green) in the patient's sample.[42,44] Interpretation of the result may be challenging and needs to take into account the size and location of the deletion or duplication, whether it has occurred de novo, and whether the region contains genes of known importance (i.e., deletion of a known haploinsufficiency gene).

4.3.6 Single Nucleotide Polymorphism Arrays

SNP arrays, which were originally designed for genotyping a patient's DNA for GWAS and cosegregation studies to determine linkage between a disease locus and a chromosomal region, can also be used to detect CNVs.[42] For example, deletions (or uniparental disomy) spanning several of adjacent SNPs included on the array will demonstrate loss of heterozygosity (LOH), while copy number gains (e.g., duplication) may be detected by the automated identification of an increased number of different genotypes.[42] The resolution of the array will depend on the number of SNPs included (and their relative spacing). Furthermore, SNP array platforms may now also include a large number of probes specifically for the detection of CNVs. SNP and aCGH arrays provide accurate tools for detecting small deletions and duplications, although they will not be able to detect balanced translocations.

4.3.7 Single Gene Testing (Sanger Sequencing)

The sequencing of a patient's DNA for abnormalities in a single or small number of genes by the Sanger method of DNA sequencing (first-generation sequencing) has been the mainstay of clinical genetic testing for monogenic disorders over the past 20 years.[16,17] In this process individual DNA fragments up to a 1000 bp in length are amplified by using the PCR, followed by a process in which a single primer is employed to initiate a reaction employing DNA polymerase to add nucleotides according to the complementary DNA strand, while randomly incorporating terminator nucleotides which harbor nucleotide-specific fluorescent labels.[16,17] This results in a mix of DNA fragments of varying length, which may be resolved by gel electrophoresis, and the sequence established from the resulting chromatogram. Sequencing reactions are typically designed to include the coding region (exons) and splice sites (intron–exon boundaries) and may also include regulatory regions [e.g., parts of the promoter, as well as 5′ and 3′ untranslated regions (UTRs)]. The high fidelity of DNA polymerase results in a high degree of accuracy such that Sanger sequencing achieves a base accuracy of >99.99%, and remains the gold standard for DNA sequencing. Indeed the advantages of this method include the ease of data interpretation and reliability of the method. However, the process is only cost effective for the evaluation of single or very small numbers of genes and is of limited value when there is weak support for a given genetic etiology.[16,17]

4.3.8 Next-Generation Sequencing or Second-Generation Sequencing

The scale, speed, and declining cost of NGS approaches has provided a paradigm shift in both the investigation and diagnosis of genetic disease and is increasingly incorporated into routine clinical care. While the principles of NGS do not differ markedly from those of Sanger sequencing (i.e., utility of DNA polymerase to incorporate fluorescently labeled nucleotides into a template DNA strand), the fundamental difference is that rather than being limited to individual DNA fragments, NGS enables simultaneous sequencing of millions of DNA fragments (i.e., "massively parallel sequencing").[16,17] Furthermore, the amount of DNA required for such a procedure is very small, so that a typical EDTA blood sample (i.e., 5–10 mL) provides far in excess of that required. The typical workflow comprises several stages: first, the DNA sample undergoes random fragmentation, followed by the addition of adapters at each end of the fragments. The resulting fragments are denatured and PCR amplified prior to immobilization on a surface by oligonucleotides complementary to the adapter sequences. The fragments then undergo amplification to form small clonal clusters, which then progress to sequencing. The sequencing process, referred to as "sequencing by synthesis", results from the incorporation of fluorescently labeled nucleotides, which enables the identification of single bases as they are incorporated into the DNA template strand.[16,17] In contrast to Sanger sequencing the incorporation of the labeled nucleotide does not terminate the reaction and thus the sequence is established through successive cycles of nucleotide addition captured by an automated camera. Typically both ends of the fragments are sequenced in a process known as "paired end sequencing," which aids the final part of the process in which the sequence reads are aligned with a reference genome.[16,17] These principles form the basis for the three most widely employed uses of NGS: WGS, whole exome sequencing, (WES) and disease-targeted gene panels.

WGS: WGS represents the most comprehensive DNA sequencing approach as it spans the entire genome including coding and noncoding regions.[16,17] While particularly suited to the identification of SNVs and small indels, it may also be used to detect CNVs although this requires increased bioinformatics expertise. Sufficient depth of coverage is required when used in the clinical setting (i.e., ~100× coverage of each base). The inclusion of all genomic regions allows the detection of mutations in both coding regions and regulatory regions (e.g., enhancers, promoters) which may not only aid diagnosis but may provide new insights into mechanisms of gene regulation. However, the inclusion of the entire genome results in several challenges, not least the huge amount of data generated and the requirement for bioinformatics expertise in its analysis. Thus, WGS remains expensive, and the frequent need to include additional family members to aid variant interpretation further increases the cost. Other limitations include difficulties sequencing parts of the genome (i.e., GC-rich regions), while highly homologous regions of DNA (e.g., due to pseudogenes) may lead to difficulties in aligning sequence reads to the reference genome. Finally, WGS will identify huge numbers of variants (i.e., ~4 million) many of them unique (i.e., never encountered in reference genomes) whose interpretation may lead to considerable diagnostic uncertainty.[16,17] The value of WGS to identify both rare coding and noncoding variants associated with skeletal phenotypes including BMD has recently been demonstrated.[24,26]

WES: In contrast to WGS, WES focuses its sequencing efforts on the 1%–2% of the genome which encodes the ~20,000 protein coding genes (i.e., the "exome") on the premise that these regions will most likely harbor disease-associated

mutations. Indeed, WES has been the mainstay of highly successful disease–gene discovery studies over the past decade, including the identification of several genes responsible for skeletal dysplasias and metabolic bone disorders [e.g., *WNT1* and *SEPINF1* mutations as rare causes of OI; *SFRP4* mutations in Pyle's disease; adaptor protein 2 sigma subunit (*AP2S1*) mutations in FHH type 3; and *PLS3* mutations in X-linked osteoporosis].[7,8,12,23,45] Thus, a major benefit of WES has been the identification of the genetic etiology of several monogenic skeletal diseases even in the absence of prior genetic linkage. Furthermore, the more manageable amount of sequence data makes analysis more straightforward, although this still may present challenges (see later). WES is achieved through an additional early step in the NGS workflow in which, following DNA fragmentation, probe sets are used to capture only the exomic regions, prior to sequencing. However, the capture may not be complete (e.g., missing exons, uneven capture due to SNPs or indels) and this needs to be considered during analysis.[16,17] Similar to WGS, GC-rich and highly homologous regions may present difficulties, while the lack of coverage of noncoding regions will exclude the identification of mutations occurring in regulatory regions. WES may also be used for the detection of large CNVs (e.g., by LOH mapping) but has deficiencies in detecting small deletions or insertions particularly if involving gene-sparse regions. Relative to WGS, WES is inexpensive and is increasingly a cost-effective approach to clinical diagnosis.

Disease-targeted sequencing: The broadest use of NGS sequencing in the clinical arena is that of targeted gene sequencing to enable the simultaneous analysis of a collection of genes specifically selected as relevant to a given disease presentation. The principle is similar to that of WES, but only specific genes of interest undergo capture with custom-designed probes.[16,17,46] Potential advantages of this approach include: the reduced cost; simplified data analysis; and the simultaneous evaluation of multiple genes that enables a rapid turnaround time, when compared to the sequential analysis of genes by Sanger sequencing. It also avoids or reduces the likelihood of several of the issues associated with WES and WGS including the identification of incidental findings (IFs) (i.e., mutations or abnormalities in genes independent from the initial reason for undertaking genetic testing). However, its limitations include the requirement to know in advance the potential causative genes (i.e., unsuitable for "gene-discovery" studies) and as the number of genes increase the likelihood of identifying variants of uncertain significance (VUSs) increases. However, the use of disease-targeted panels continues to gain increasing traction with many now offered in the field of skeletal dysplasia and metabolic bone disease (registered diagnostic genetic testing providers may be found at: https://www.ncbi.nlm.nih.gov/gtr/). For example, NGS disease-targeted panels have been established for OI, skeletal dysplasia, high and low BMD phenotypes, mineralization defects including hypophosphatemic rickets, and many others.[14] The number of genes included in such panels varies considerably from <10 to >150 genes. The utility of such panels depends on the clinical scenario. For example, when referred a child for whom OI is suspected because of severe skeletal fragility yet no *COL1A* or *COL1A2* mutation has been detected, additional genetic testing employing a skeletal-fragility gene panel may provide a useful and cost-effective approach.[5,14]

4.4 Challenges of Data Interpretation and Approaches to the Analysis of Variants Identified by NGS Platforms

A major challenge arising from the increasing clinical and research application of genetic testing, including the use of each of the main NGS platforms, is accurate data interpretation. For example, WES and WGS may require the analysis of hundreds of SNVs, while disease-targeted gene panels may also identify several different SNVs in plausible candidate genes, and in each case the relevance to the clinical phenotype needs to be determined. In these settings, ascribing pathogenicity to individual variants may be challenging and as a result, guidelines have been recommended by several international bodies suggesting a systematic approach to variant analysis. These guidelines recommend that all variant classification systems utilize the terms "pathogenic," "variant of uncertain significance (VUS)," and "not pathogenic or benign," and that evidence for pathogenicity is gathered from multiple sources.[47,48] In addition, an increasing number of computational tools are now available to aid the analysis of variants, which can be utilized both in the clinical and research setting, although an over-reliance on such tools in the absence of robust clinical and/or functional support may result in inaccurate estimates of pathogenicity. These potential considerations and processes that are typically included in the variant identification and interpretation pipeline are briefly reviewed, below.

4.4.1 Variant Identification

For all NGS platforms, sequencing is followed by a series of analyses to map the data and identify variants. This begins with the alignment of the sequence reads to a reference genome, which should be obtained from a

reputable source, such as the Genome Reference Consortium human genome (GRCh). Several tools can be utilized to align sequences to reference genomes (e.g., BWA-MEM, Bowtie2, and Novoalign) and a combination of these tools is recommended to improve accuracy.[49] These tools estimate the probabilities of possible genotypes at each locus, based on quality scores of the reads and the number of times an allele has been observed. As such, a high depth of coverage is advantageous in accurate alignment. Annotation of SNPs is then performed using variant calling tools, of which the Genome Analysis Tool Kit HaplotypeCaller (GATK-HC), Samtools mpileup, Freebayes, and Torrent Variant Caller are widely used.[49] However, there may be discrepancies between these tools, including biases toward the detection of certain variant types, which may result in false positive annotations. Thus, employing a combination of tools is recommended, and caution is required when examining results, such that variants identified as pathogenic or to be further investigated should be verified by an alternative sequencing technique, like Sanger DNA sequencing.

4.4.2 Variant Interpretation

Following the identification of one or more SNVs from the initial variant calling algorithm, further analysis is required to identify those that may be relevant to the disease phenotype. Although the pipeline used to interrogate variants will vary according to whether the sequencing is being performed for clinical testing of known disease genes (e.g., OI or skeletal dysplasia gene panels), or for exploratory studies to investigate novel phenotypes, the principles for ascribing potential pathogenicity are similar (Fig. 1.2). In most instances it is reasonable to exclude all synonymous variants from further analysis (i.e., those not changing the amino acid sequence of the encoded protein). The initial analysis of nonsynonymous variants should consider and report the frequency of the variant in large population databases including dbSNP, 1000 Genomes, Exome Aggregation Consortium, and the Exome Variant Server.[47,48,50,51] A frequency of >1% in such databases indicates that the variant is a likely benign polymorphism and therefore unlikely to be pathogenic. This is particularly pertinent in rare diseases in which the frequency of a variant in the population database should not exceed that within the disease population.[50] Furthermore, for disorders that are fully penetrant, the presence of the identified variant at a frequency of >1% within control population databases indicates that it is unlikely to be disease causing.[47,50] However, the presence of variants at low frequencies (<1%) in these databases does not exclude their pathogenicity as the databases may contain representative individuals from the disease population (e.g., The Cancer Genome Atlas).[47] In addition to these population databases, clinical variant, and disease databases (e.g., ClinVar, Human Gene Mutation Database) should be consulted to determine if the gene has previously been linked with the condition.[51] A degree of caution must be exercised with many such databases because robust support for the pathogenicity of variants is often absent, and many do not undergo regular curation leading to inaccuracies. Where possible the primary source of information supporting previous reports of variant pathogenicity should be consulted.[47,51] Meanwhile, determining whether the variant segregates only with affected family members is key to attributing disease causality. Segregation in close family members is not evidence of causality per se, as the variant may actually be in linkage disequilibrium with the causative gene, and thus samples should be sought also from distant family members if possible. Likewise, in the research setting, prior cosegregation studies in a family, which may have identified putative chromosomal locations shared by affected individuals, may markedly narrow the focus on specific variants.

Further investigation of variants for likely functional effects relies on an assessment using computational tools and/or employing in vitro and or in vivo assays. Thus, variants can be subdivided into: those that are missense, nonsense, or frameshift variants; those within exons and introns predicted to affect splice sites; and those within UTRs. One can then assume that nonsense, frameshift, and splicing mutations that lead to whole exon deletions are more likely to be pathogenic. However, the biological function of the gene must also be considered as some diseases are only caused by heterozygous missense mutations, while many genes harbor high frequencies of heterozygous null variants without any detrimental effect on health.[47] Similarly, truncating or frameshift mutations that affect the extreme 3′ end of a gene are less likely to be pathogenic as key functional domains of the protein may be retained.[47,48] For splice-site variants, the variant may lead to exon skipping, exon truncation, or use of an alternate donor or acceptor site and a number of freely available programs may be employed to predict whether a coding or intronic variant is likely to influence splicing (Alternative Splice Site Predictor, The Human Splicing Finder), although confirmation of a functional effect is usually required to verify this. For variants in UTRs, the possibility that there is disruption of a promoter or microRNA binding site should be considered, and this can be predicted using online tools (mirdb, UCSC Genome Browser).[47,48]

For missense variants a number of computational predictive software packages examine different parameters; for example, SIFT uses evolutionary conservation, Polyphen-2 and MutationTaster use combined protein structure–function and evolutionary conservation, while Provean predictions are based on multiple

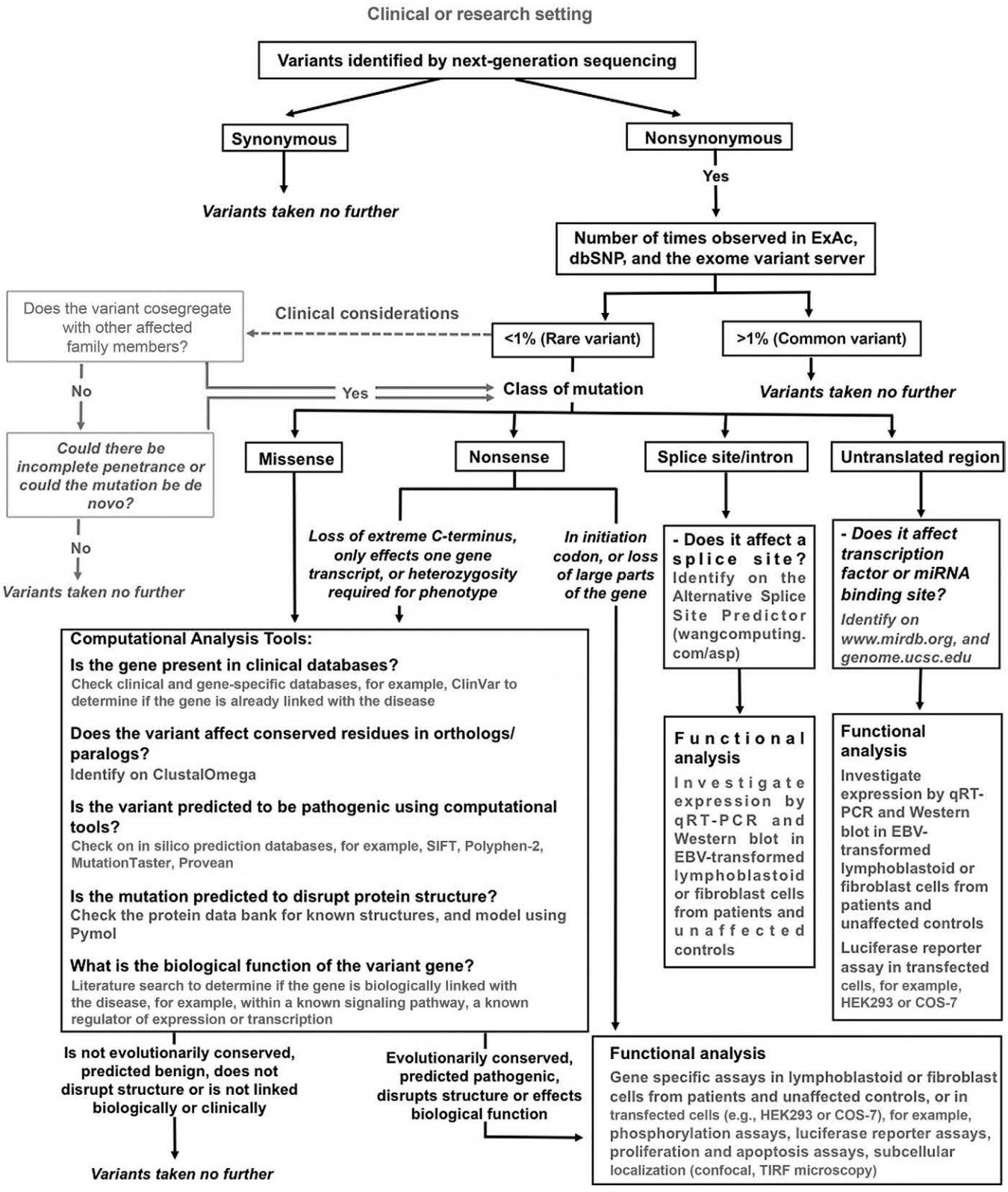

FIGURE 1.2 **Flow diagram outlining methods to assess the potential pathogenicity of genetic variants identified by next-generation sequencing (NGS).** Within the clinical or research setting, variants identified by NGS platforms require a systematic approach to ascribing potential pathogenicity. Initially, single nucleotide variants (SNVs) are broadly categorized into synonymous or nonsynonymous variants, with synonymous variants typically excluded from further analysis. For evaluation of nonsynonymous variants, an evaluation of the population frequency is an essential step, typically allowing the exclusion of variants which are observed with an allele frequency of >1%. Concurrently, it is important to consider the clinical evidence for the pathogenicity by evaluating whether the variant cosegregates with affected individuals in the kindred (i.e., all affected should harbor the variant) or occurs in regions shared by affected individuals from prior positional cloning studies. Variants are then divided by functional type into missense, nonsense, splice site variants, or those affecting untranslated regions (UTRs), as approaches to analyses differ for each variant class. For missense and nonsense variants, a number of computational analysis tools exist to examine frequency in clinical populations, evolutionary conservation and the effect on protein structure. While these can be informative, they should not be used as the only indicator of pathogenicity as they have low specificity. In the absence of very strong support for pathogenicity (e.g., nonsense mutation in gene in which similar mutations previously confirmed as disease causing, or clear enrichment for variant in disease cohorts relative to controls) variants suspected of being pathogenic should be analyzed functionally using an appropriately designed assay.

sequence alignment.[47] Although these algorithms can assess the pathogenicity of a variant, studies of known disease-causing variants using in silico predictive tools have shown that this is only 65%–80% accurate.[52] Thus, protein-prediction software cannot be the sole provider of evidence supporting pathogenicity in the clinical or research settings. Identification of evolutionary conservation or location of the variant residue within a critical structural domain of the affected protein can also provide evidence that a variant may be pathogenic. Several computational tools are available to assess these parameters including ClustalOmega that can align sequences based on homology, the Protein Data Bank that catalogs published protein structures, and Pymol that can be used to model structures downloaded from Protein Data Bank. In the research setting, an important consideration in the analyses of variants identified by NGS in genes not previously associated with disease is establishing the plausibility of the gene being disease causing. A literature search should be performed to determine the biological function of the affected gene. For example, if the gene is known to regulate a signaling pathway already known to be important in the condition (i.e., Wnt or OPG/RANKL/RANK/NF-κB pathway in many skeletal disorders), or if the gene regulates expression or transcription of pathway genes, this provides support to the likely pathogenicity.

Once a variant has been assessed by these bioinformatics tools and still remains a plausible candidate, functional analysis should be performed. Some care must be taken to select the appropriate experimental technique and this may depend on: the type of variant (e.g., splice site variants are best assessed by measurement of RNA and protein expression, while missense mutations may require functional cellular assays); the tissue affected in the disease; and the availability of samples.[53] Ideally assessments should be made in patient tissue where possible, for example in cell lines derived from lymphoblastoids or skin fibroblasts, or histological sections of tissues removed by biopsy or surgery. In choosing an in vitro assay the heritability of the variant should also be considered as overexpression systems may exaggerate the effect of a variant, and therefore may not accurately reflect the effect of a heterozygous variant. Further important evidence to implicate a gene causing a disease can come from screening additional affected families and individuals for further mutations in the gene.

4.5 Special Circumstances for Genetic Testing

4.5.1 *Detection of Mosaicism*

Mosaicism may arise by a number of genetic mechanisms, and its detection requires specific consideration.[54] Mosaicism refers to an individual who has developed from a single fertilized egg but has two or more populations of genetically distinct cells. Typically, this involves a postzygotic de novo mutational event (e.g., aneuploidy, CNV, SNV) leading to the distinct population of cells, and a given phenotype. While certain disorders only manifest as mosaicism (e.g., McCune–Albright syndrome), another class of mosaic disorders is those harboring mutations more frequently associated with autosomal dominant disorders. This includes some individuals with relatively mild OI, as well as others with "segmental" neurofibromatosis type 1 in which the manifestations are limited to a single region of the body presumably due to somatic *NF1* mutation during organogenesis.[54] Germline mosaicism, arising from somatic mutation during gametogenesis, also may lead to diagnostic and recurrence risk confusion. In this setting, apparently unaffected parents (with one carrying the mutation limited to their gametes) may give rise to more than one affected child, suggesting possible autosomal recessive inheritance, in contrast to the underlying autosomal dominant inheritance pattern. For example, germline mosaicism has been reported in OI type II, in which apparently unaffected parents can have recurrently affected babies.[54]

The ability to detect mosaicism has evolved with the development of improved genome-wide genetic testing including aCGH, SNP arrays, and NGS. Specifically, these tests provide far greater sensitivity to detect low levels of mosaicism (e.g., 5% for SNP array) when compared with traditional approaches (e.g., karyotyping, Sanger sequencing). However, the most appropriate diagnostic test will depend on the clinical picture, the suspected type of mutation (e.g., aneuploidy, CNV, SNV, "indel") and the likely extent of mosaicism. Often lymphocyte DNA will suffice, but this will depend on the affected tissue(s). For example, exome sequencing of affected biopsy tissue from individuals with the Proteus syndrome, which is the disease that probably affected Joseph Merrick "The Elephant Man" and is characterized by overgrowth of skin, bone, and other tissue, revealed its genetic etiology to be due to somatic mosaic mutations in an oncogenic transforming (T) gene originally derived from mice referred to as stock A and strain K theme 1 (*AKT1*).[54,55]

4.5.2 *Prenatal Diagnosis*

The ability to undertake prenatal genetic diagnosis may be important for families at risk of transmitting heritable disease.[56] This may include potential parents: with a specific genetic disorder or those who are known carriers of disease-associated mutations; with previously affected children with genetic disease; or in whom routine antenatal screening (e.g., ultrasonography) raises the possibility of a genetic skeletal or

metabolic disorder. The potential indications, nature, and timing of genetic testing will therefore depend on the clinical scenario.

Preimplantation genetic diagnosis: For individuals at risk of a serious genetic disorder, in vitro fertilization offers the opportunity to screen embryos for the specific defect prior to establishing pregnancy. In this process, a few days after egg fertilization, a single cell is removed from the embryo for genetic analysis, which allows the detection of major chromosomal abnormalities (i.e., by aCGH or FISH) or the evaluation for single gene defects (i.e., conventional PCR and sequencing). Screening of serial embryos allows up to two unaffected embryos to be subsequently implanted for pregnancy to develop.
Prenatal genetic testing: If pregnancy is already established the ability to identify fetuses at risk of genetic disease traditionally involves invasive genetic testing through chorionic villous sampling (CVS) or amniocentesis. However, recent progress in the detection of cell-free circulating fetal DNA in the maternal circulation now offers the potential for noninvasive prenatal genetic diagnosis (NIPD) and/or testing (NIPT).[56]
Invasive genetic testing: Typically prenatal genetic testing has been performed on cells obtained from CVS or amniocentesis samples. These samples have been used in tests that include: conventional karyotyping for the detection of aneuploidy (i.e., Down's syndrome); FISH or aCGH for the detection of alternate chromosomal abnormalities (e.g., deletions, duplications); and DNA sequence analysis to test for single gene defects responsible for monogenic disorders. In theory, DNA samples may be evaluated by NGS approaches (e.g., gene panel, WES) but experience in the prenatal setting remains limited. While these tests may reliably identify the genetic risk to the fetus, the CVS and amniocentesis procedure carry potential risks to the pregnancy (e.g., miscarriage, infection), which need to be discussed prior to testing.
NIPD/NIPT: While the majority of the cell-free DNA in the maternal circulation arises from the mother, 10%–20% arises from the placenta, and the ability to detect this cell-free fetal DNA provides the basis for NIPD/NIPT, which involves a maternal blood test at an appropriate stage of gestation (e.g., after 10 weeks).[56] Such clinical use of NIPD/NIPT is a recent development, although it has a growing number of potential applications. Currently, in the clinic it is most frequently used to screen for aneuploidy (Down, Edwards, Patau, and Turner syndromes) and for fetal sex determination (important for X-linked disorders or conditions that severely affect one sex, for example, females with congenital adrenal hyperplasia). However, it is increasingly offered on a clinical basis for the detection of monogenic disorders including severe skeletal dysplasia, although currently its clinical use is limited to assess for paternally inherited mutations or those arising de novo as it is not possible to distinguish abnormalities inherited from the mother (due to the presence of maternal cell-free DNA in the sample).[56] In the research setting, a great number of potential applications for cell-free fetal DNA are being evaluated, including the ability to determine the entire fetal genome, enabling both the detection of CNVs and SNVs although further optimization of these methods is required before being implemented in a clinical setting. At the same time, the prospect of such technology raises important ethical issues.

4.6 Informed Consent and Ethical Considerations

Establishing a genetic diagnosis may benefit the patient and family. However, such genetic testing may also give rise to clinical and ethical challenges.[47,50,53,57] For example, the increasing implementation of genome-wide approaches (e.g., aCGH, SNP arrays, WES, WGS) has led to far better understanding of the degree of rare genetic variation in the population including both SNVs and CNVs.[50] Nevertheless, our current ability to accurately interpret genome-wide data sets remains limited, and in many circumstances diagnostic uncertainty may persist after such genetic testing. Thus, multiple gene testing or whole genome approaches employing NGS inevitably identifies variants of uncertain significance (VUSs). Communicating this uncertainty to the patient (and perhaps the wider family) remains a major clinical challenge. However, it is clear that the likelihood of such ambiguous results should constitute part of informed consent prior to genetic testing. Recent studies have also demonstrated that for several disorders many variants reported as pathogenic may instead be either benign or far less penetrant than previously recognized, and the potential implications of such misclassifications for patients and family members may be substantial (i.e., through inappropriate investigation or treatment, or decisions regarding conception).[47,50,57] Thus, caution is required in data interpretation and both the clinician and patient must have an understanding of the genetic test result and its potential limitations. In addition, clinicians and academics both have a responsibility for accurate reporting of genetic data coupled with phenotype data to improve the reliability of disease-specific mutation databases.[47,53] Plans to deal with IFs, which are genetic abnormalities not related to the clinical question but of potential clinical significance to the patient and

their family, also need to be considered. For example, the inadvertent identification of penetrant pathogenic mutations in hereditary cancer genes (e.g., *BRCA1*, *BRCA2*) may result in complex ethical considerations, and plans for managing these findings appropriately should be in place and be a key part of the informed consent pathway.

5 CONCLUSIONS

Many skeletal and mineral metabolic diseases have a genetic basis, which may be a germline single gene abnormality (i.e., a monogenic or Mendelian disorder), a somatic single gene defect (i.e., a postzygotic mosaic disorder), or involve several genetic variants (i.e., oligogenic or polygenic disorders). Recognition of these heritable disorders is clinically important, as it can facilitate relevant and timely investigation and treatment for the patients and families. Recent advances in genetics and DNA sequencing methods have resulted in new ways to detect genetic abnormalities, making it increasingly important for the clinician to gain an appreciation of these complex tests and to couple this with the fundamental skills of history taking and physician examination to ensure appropriate and judicious use of these genetic tests for the benefit of patients.

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CHAPTER

2

Epigenetics

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1 INTRODUCTION

Genetic information is stored in DNA and faithfully transmitted from one cell to its daughter cells and to the next generation through the germline. Genetic changes are irreversible alterations of the DNA sequence that are heritable somatically and through the germline. These alterations include single nucleotide variants or polymorphisms, tandem repeats (short tandem repeats or variable number of tandem repeats), small indels (insertions or deletions of short DNA sequences), large deletions or duplications that alter the copy number of DNA segments, and chromosomal rearrangements associated with inversions and translocations (copy-neutral variations).[1] A vast majority of the genetic variations observed in the human population are well tolerated and do not adversely affect phenotype or normal functions. Genetic variations that cannot be tolerated can cause susceptibility to various disease states and abnormal functions. Genetic linkage studies and targeted sequencing together with recent advances, such as genome-wide association study and next-generation sequencing approaches have provided valuable insight into normal and abnormal genetic variations (see Chapter 3).

Translation of the same genetic information (genotype) to produce cell-type-specific phenotype is achieved by epigenetic control mechanisms that establish, regulate, and maintain specialized gene expression programs without altering the underlying DNA sequence. In 1942, the developmental biologist Conrad Waddington used the term "epigenetic landscape" to describe the phenotypic alterations that occur from cell to cell during development in multicellular organisms (this article from 1942 was reprinted in 2012).[2] From biochemical studies and experiments conducted in model organisms and human cells we now know that epigenetic control is achieved by changes to posttranslational modifications (PTMs) of histones, chromatin structure, DNA methylation state, and noncoding RNA interactions.[3] Therefore, epigenetics has been molecularly defined as "the sum of the alterations to the chromatin template that collectively establish and propagate different patterns of gene expression (transcription) and silencing from the same genome."[4] Epigenetic changes are retained through successive cell divisions and evidence also suggests transgenerational transmission, particularly of DNA methylation associated with imprinting. Unlike genetic alterations epigenetic changes are reversible, a property that is desirable for the development of drugs that can combat disease states associated with epigenetic defects. This chapter presents a general background of epigenetic control mechanisms and the contribution of epigenetics to human disease.

2 EPIGENETIC CONTROL MECHANISMS

DNA in eukaryotic cells is packaged and organized into chromatin. Nucleosomes are the basic units of chromatin. Each nucleosome consists of 147 bp of DNA wound almost twice around a histone octamer containing two copies each of the four histone proteins H2A, H2B, H3, and H4.[5–7] In addition, linker histones (histone H1 family) are incorporated where the DNA enters and exits the nucleosome. Nucleosomes are assembled in an array connected by 10–70 bp of linker DNA, often described as "beads on a string." This DNA compaction surrounded by histones as well as the folding of the nucleosomal arrays acts as a barrier to enzymes and other factors that require access to DNA to affect gene transcription and DNA replication and repair. Various epigenetic control mechanisms cooperate to regulate gene "ON" and "OFF" states by affecting access to DNA by transcription factors and the general transcriptional machinery and posttranscription regulation of RNA, and help to maintain genome integrity. These mechanisms include posttranslational modifications of histones, chromatin remodeling, DNA methylation, and noncoding RNA interactions.

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2.1 Histone Modifications and Chromatin Remodeling

Histone proteins are highly conserved from yeast to humans. They contain a globular domain and a flexible unstructured amino terminal tail (the histone tail) that protrudes from the nucleosome. Many amino acid residues on the histone tails and some residues in the globular domain, particularly of histone H3 and H4, undergo covalent PTMs contributing to chromatin variability. Several different histone PTMs and the corresponding, enzymes that catalyze or reverse these modifications have been characterized, including acetylation, methylation, phosphorylation, ubiquitination, ADP-ribosylation, crotonylation, GlcNAcylation, and others that are being functionally characterized (Table 2.1, Fig. 2.1).[8,9] Mechanisms of reversible incorporation of acetyl, phosphate, and methyl groups within histone tails are best understood. Specific enzymatic activities that reside in large multisubunit complexes govern the steady state balance of each modification such that each is established (written), removed/reversed (erased), or recognized (read).[10]

Histone acetyl transferases acetylate specific lysine residues in histones that are reversed by histone acetyl deacetylases (HDACs).[11] Histone acetylation often correlates with active chromatin regions that are accessible to the transcriptional machinery. Histone acetyl transferases belong to five different families, of which members of the Gcn5-*N*-acyltransferases and MYST families are the most abundant, followed by the steroid receptor coactivator family and the activating transcription factor (ATF-2) and TAFII250 families. Sequence homology among the families is low. However, they all show structural similarity in the core enzymatic region. Mammalian HDACs have been categorized into four major classes, class-I: HDACs 1, 2, 3, and 8; class-IIa: HDACs 4, 5, 7, and 9; class-IIb: HDACs 6 and 10; class-III: the sirtuins (SirT1-7); and class IV, HDAC11.

Enzymes of the histone kinase family phosphorylate specific serine, threonine, or tyrosine residues, and phosphorylation can be reversed by the action of phosphatases.[12] Phosphorylation of histone amino acid residues have been observed at condensed chromatin associated with transcriptional silencing.

Histone lysine methyltransferases (KMTs) are SET domain and non-SET domain-containing methyltransferases that methylate lysine residues that can be removed by histone lysine demethylases (KDMs).[13] Protein arginine methyltransferases methylate arginine residues that can be indirectly removed by arginine deiminases that convert methyl-arginine to a citrulline residue.[13,14] Lysine methylation can be present in mono-, di-, or trimethylated states. For example, di- or trimethylation of histone H3 at lysine 4 (H3K4me2 and H3K4me3) and monomethylation of H3K9 (H3K9me1) are marks of active transcription. However, di- and trimethylation of H3K9

TABLE 2.1 Histone Posttranslational Modifications

Modifications	Abbreviations	Amino acids
Acetylation	Ac	K, S, T
ADP ribosylation	Ar	K, E
Butyrylation	Bu	K
Citrullination	Cit	R
Crotonylation	Cr	K
Formylation	Fo	K
2-Hydroxyisobutyrylation	Hib	K
Hydroxylation	OH	Y
Malonylation	Ma	K
Methylation	Me	K, R
O-GlcNAcylation	Og	S, T
Phosphorylation	Ph	S, T, Y, H
Propionylation	Pr	K
Succinylation	Su	K
Ubiquitination	Ub	K

Histone modifications from Huang H, Sabari BR, Garcia BA, Allis CD, Zhao Y. SnapShot: histone modifications. Cell *2014;***159**(2)*:458.*[9]

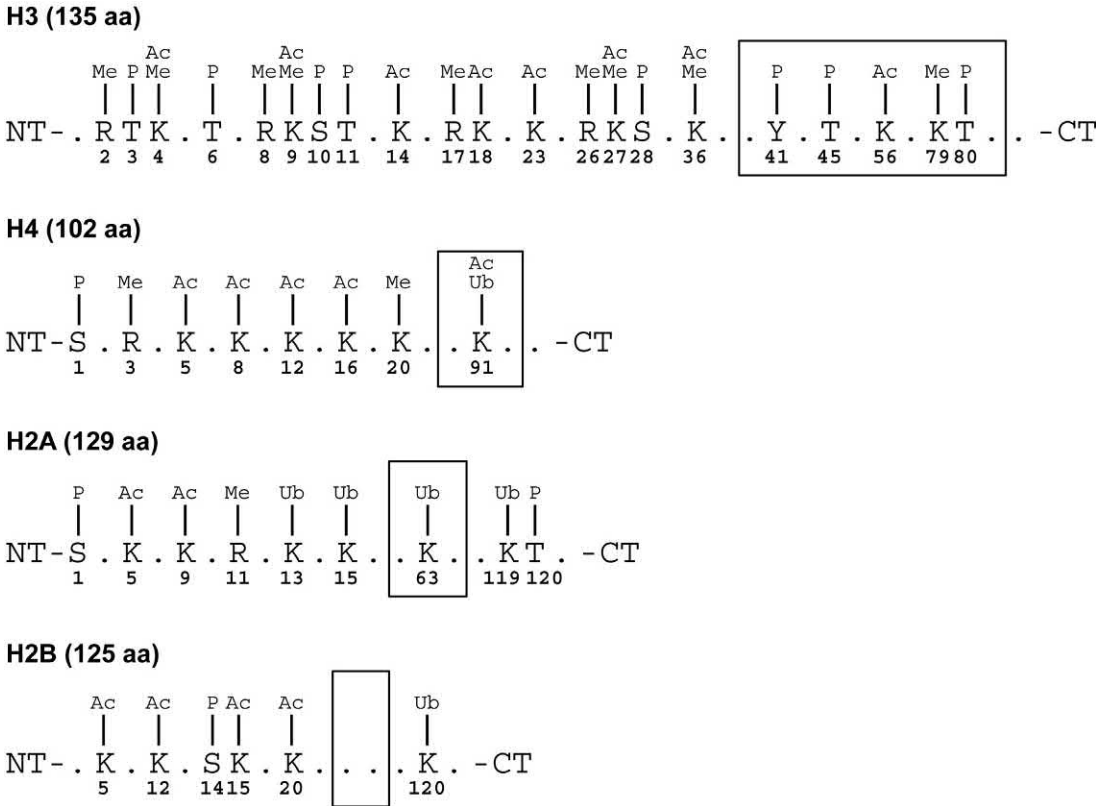

FIGURE 2.1 Histone posttranslational modifications (PTMs). Commonly observed and well-studied histone PTMs of the four canonical core histones are shown: acetylation (Ac), methylation (Me), phosphorylation (P), and ubiquitination (Ub).[4,9] Only the amino acids that are modified are shown. The amino acid position in the histone protein sequence is written below each amino acid. The boxed area in each histone marks the globular domain. NT and CT are N and C terminus, respectively, of each histone protein.

(H3K9me2 and H3K9me3), and H3K27 (H3K27me2 and H3K27me3) are repressive marks that block gene expression. Lysine-specific histone demethylase 1 (LSD1 or KDM1A) and lysine-specific histone demethylase 2 (LSD2 or KDM1B) remove mono- and dimethylation of H3K4 and H3K9. Another family of KDMs shares the jumonji C domain (JMJC) (JHMD1, JMJD3, and JMJD2D) to mediate demethylation, each targeting specific methyllysine groups.

Various combinations of PTMs can occur on the same histone tail (in *cis*), or on another tail (in *trans*), that constitute a "histone code" or a signature.[10] This code can be deciphered by proteins, which contain binding motifs specific for each modification (readers) that are part of reader-containing complexes, eventually resulting in chromatin remodeling.[15] Reader domains of specific methyllysine residues are the best-characterized group and include ATRX-DNMT3-DNMT3L (ADD), ankyrin, bromo-adjacent homology (BAH), chromobarrel, chromodomain, double chromodomain (DCD), malignant brain tumor (MBT), plant homeodomain (PHD), Pro-Trp-Trp-Pro (PWWP), tandem Tudor domain (TTD), Tudor, WD40, and the zinc finger CW (zf-CW). Acetylated residues are read by proteins that contain PHD or by the bromodomain extraterminal (BET) motif. The BRCT and BIR domains can bind to specific phosphorylated residues. Reader-domain containing subunits are essential for ATP-dependent nucleosome-remodeling complexes, such as switching defective/sucrose nonfermenting (SWI/SNF) for binding and remodeling activity to slide or displace histones, working at promoters and enhancers to regulate transcriptional responses.

The polycomb group (PcG) and trithorax group (TrxG) proteins are evolutionarily conserved regulators that maintain transcriptional "OFF" and "ON" states, respectively, of development control genes, such as *HOX* and other target genes.[16] They function to remodel chromatin in concert with chromatin-modifying enzymes by altering the accessibility of DNA to factors required for gene transcription.[17,18] PcG proteins consist of two separate multimeric protein complexes that are associated with repressed chromatin states, such as the PcG repressive complex 1 (PRC1) and the PcG repressive complex 2 (PRC2). PRC1 monoubiquitinates histone H2A on lysine 119 (H2AK119Ub1). PRC2 trimethylates histone H3 at lysine 27 (H3K27me3) through the activity of PRC2 component enzymes EZH1 or EZH2 (enhancer of zeste homolog 1 or 2) in association with EED or SUZ12. TrxG proteins also exist as several multimeric protein complexes containing histone methyltransferase

activity that are associated with active chromatin states where they induce trimethylation of histone H3 at lysine 4 (H3K4me3). Therefore, the functions of the PcG and TrxG proteins are described as "antagonistic" to each other promoting transcription repression and activation, respectively.

2.2 Histone Variants

During the process of transcription elongation, nucleosomes obstruct the passage of RNA polymerase (Pol) II or RNA Pol I. Chromatin remodeling activity facilitates transcriptional initiation and elongation allowing the passage of the RNA polymerase through nucleosomal arrays by displacing histone H2A/H2B dimers, which can lead to the exchange of core histones with histone variants.[19] All of the core/canonical histones except histone H4 have variant isoforms (nonallelic isoforms of the canonical histone proteins) that differ by a few amino acids. They localize on the newly synthesized daughter strands following DNA replication and also play an important role in regulating gene expression (a replication-independent function). The histone H1 family includes multiple isoforms; H2A variants include H2A.Z, MacroH2A, H2A-Bbd, H2AvD, and H2A.X; and H3 variants include H3.3 and centromeric-specific isoform (CENP-A). Histone variants and their modifications contribute to nucleosome and chromatin structure and function.[8] For example, transcriptionally active genes exchange the canonical H3 with the H3.3 variant contributing to the active epigenetic signature.[20] Also, the incorporation of H2A.Z together with H3.3 leads to an unstable chromatin state associated with open chromatin at enhancer elements.[21]

2.3 DNA Methylation

One of the most common epigenetic control mechanisms associated with gene repression/silencing is DNA methylation at cytosine residues.[22,23] This modification mainly occurs at CpG dinucleotides where a cytosine nucleotide is followed by a guanine nucleotide in which cytosine is converted to 5-methylcytosine (5mC). CpG islands are DNA regions up to several hundred base pairs in length that are CG-rich and that contain many CpG sites. CpG islands often occur in promoter regions and their methylation usually prevents gene expression. Emerging evidence shows that DNA methylation can also occur at nonCpG sites (CpA, CpT, and CpC) and may control gene expression.[24]

Cytosines are methylated by DNA methyltransferases (DNMT3A and DNMT3B). This modification is maintained during DNA replication and can be passed on after cell division to daughter cells. DNA replication leads to hemimethylated CpGs and the methylation is maintained by DNMT1 that methylates the CpG on the unmethylated strand.[23] DNMT3A and DNMT3B are responsible for de novo DNA methylation and associate with DNMT3L that is a catalytically inactive DNA methyltransferase required for DNA methylation. DNMT3L recognizes unmethylated histone H3 lysine 4 (H3K4) to induce DNA methylation by recruitment or activation of DNMT3A and DNMT3B. On the other hand, the presence of H3K4me3 protects CpG islands from DNA methylation by inhibiting DNMT3L binding. Thus there is an antagonism between H3K4me3 and DNA methylation.[25] Studies in mouse models have shown that *Dnmt* genes are essential for development as targeted deletion of *Dnmt* genes leads to early embryonic lethality.[26,27]

DNA methylation is reversible in that 5mC can be converted to 5-hydroxymethylcytosine (5hmC) by the ten-eleven translocation (TET) family of methylcytosine hydroxylases.[28,29] The hydroxylase activity of the TET family of 5mC hydroxylases (TET1, TET2, and TET3) promotes DNA demethylation by converting 5mC to 5hmC and further to 5-formylcytosine (5fC) and finally to 5-carboxylcytosine (5caC). Further reactions, such as decarboxylation of 5caC could then lead to DNA demethylation and conversion back to cytosine. Whether these intermediates of DNA demethylation by themselves have functional significance in epigenetic control is being actively investigated.[23] Drugs, such as 5-azacitidine (5AC) and 5-aza-2′-deoxycytidine (DAC) can inhibit and degrade DNMTs to block DNA methylation.

How DNA methylation functions to silence gene expression is not clearly understood. There is some evidence for transregulation through methyl-CpG binding (MBD) domain proteins, such as methylcytosine-binding protein 2 (MeCP2) that binds to methylated cytosines to recruit HDACs thereby removing the gene activation-associated acetylation marks.[23] Also, DNA methylation has been shown to silence gene expression by affecting the recognition sites of transcription factors or other transcriptional regulators.[30]

Allelic differences in DNA methylation are seen at imprinted loci to differentially silence the paternal or the maternal allele, and at the inactive X-chromosome.[30] Such differentially methylated regions (DMRs) in the genome are also capable of controlling gene expression of multiple genes associated with the DMR, such as at the *SNRPN-UBE3* and *DLK1-MEG3* gene clusters.[30] Defects in DNA methylation have been observed in several human disorders, such as disorders of genomic imprinting and cancer.[31,32]

2.4 Noncoding RNAs (ncRNAs)

The draft sequence of the human genome published in 2001 and more recent data estimate the presence of ~21,000 protein-coding genes encoded by the DNA.[33,34]

A large proportion of the genome is transcribed into small RNAs or long noncoding RNAs (lncRNAs) that lack open reading frames for translation into proteins. Some of these RNAs have been shown to silence gene expression by triple helix formation with DNA or base-pairing at target regions, by acting as a scaffold for chromatin-modifying and -remodeling protein complexes, and also by recruiting factors, such as the PcG complexes that silence the chromatin or factors that facilitate transcription.[35] The long intergenic ncRNA (lincRNA) X-inactive specific transcript (Xist) is expressed from one female X-chromosome as a 17 kb polyadenylated noncoding RNA. Xist "coats" the X chromosome from which it is transcribed and leads to the recruitment of PcG proteins, such as PRC2 to silence transcription across this chromosome in *cis* (X-chromosome inactivation).[36] Another lincRNA Hox transcript antisense intergenic RNA (HOTAIR) transcribed from the *HOXC* cluster silences the *HOXD* cluster located on another chromosome in *trans* by physically interacting with the PRC2 complex and targeting PRC2 to the appropriate target site.[37] Similarly, several other lncRNAs have been shown to associate with PRC2 to localize the epigenetic regulatory machinery at target sites in *cis* or *trans*.[38,39]

Two classes of small RNA, short (or small) interfering RNA (siRNA) and microRNA (miRNA) play a central role in RNA interference-mediated gene silencing mechanisms.[40] They differ in biogenesis, processing, and mechanism of action. The precursors of siRNA and miRNA are double-stranded RNA structures formed due to base pairing of complementary RNAs, or from intramolecular RNA folding into stem-loop hairpin structures, respectively. After a series of processing steps an RNase III class ribonuclease, Dicer, cleaves the double-stranded RNA molecules into siRNA or miRNA. These processed small RNAs, 21–24 nucleotides in size, associate with effector protein complexes that contain Argonaut and other factors for gene silencing by targeting either messenger RNAs posttranscriptionally for degradation or chromatin regions through complementary base-pair interactions, or by blocking translation.[40,41] Two related complexes that incorporate siRNA or miRNA, RNA-induced silencing complex (RISC) and RNA-induced transcriptional silencing (RITS) complex target messenger RNAs and chromatin, respectively.[41]

3 TRANSGENERATIONAL EPIGENETIC INHERITANCE

The structure of the DNA double helix and selfpropagation of DNA through semiconservative replication allows for storage and maintenance of both normal and abnormal genetic information, and transmission to daughter cells somatically and to the next generation through the germline. Epigenetic modifications can be passed on to daughter cells somatically after cell division.[42] However, a vast majority of the epigenetic modifications reset during meiosis. This reprogramming resets the epigenome (epigenetic marks across the entire genome) of the early embryo to render the cells totipotent (the potential to form every cell type in the body). To pass to the next generation through the germline, epigenetic marks must avoid being erased during embryonic reprogramming.[43] Imprinted genes have been shown to bypass epigenetic reprogramming postfertilization. Recent work shows that the DNA binding factor Zfp57 together with Kap1/Trim28 may be critical for postfertilization maintenance of maternal and paternal DNA methylation imprints.[44,45] The precise mechanisms that allow transgenerational retention of DNA methylation imprints and other epigenetic modifications are being investigated.[43]

4 EPIGENETICS AND HUMAN DISEASE

Epigenetic changes have been observed in many disease states that can occur as a result of mutations in factors that control epigenetic modifications (writers, erasers, or readers) or in response to environmental stimuli (endogenous or exogenous signals). Such epigenetic changes can be the cause or consequence of the disease process serving as disease drivers, biomarkers, and therapeutic targets.

4.1 Imprinting Disorders

Most genes show a biparental (diploid) mode of expression. However, a subset of "imprinted" genes are expressed in a monoallelic parent-of-origin dependent manner in which one parental allele is partially or completely silenced. Some of the imprinted genes have been well characterized.[30] Paternally expressed imprinted genes that normally function as growth promoters include *DLK1*, *IGF2*, *PEG1*, *PEG3*, and *RASGRF1*. Maternally expressed imprinted genes that normally function as growth repressors include *CDKN1C*, *GNAS*, *GRB10*, *H19*, and *IGF2R*. Another class of imprinted genes are associated with behavioral and neurological defects, such as *KCNQ1*, *NESP*, and *UBE3A*. Imprinted genes are located in clusters where the parental alleles possess differential DNA methylation marks at DMRs and other epigenetic modifications at imprinting control elements.[46] Tissue-specific imprinting (silencing of one allele in a tissue- or temporal-specific manner) occurs in some genes (e.g., *GNAS*) (see Chapter 35). Imprinting disorders can result from mutations in the nonsilenced allele or from DNA methylation defects, such as loss of

imprinting causing two active copies of a gene or from inactivation of the nonimprinted allele causing two inactive copies.[30] Such conditions can lead to severe developmental abnormalities, cancer, and other diseases. Some of the well-characterized imprinting disorders include Prader–Willi syndrome, Angelman syndrome, Beckwith–Wiedemann syndrome, Silver–Russell syndrome, Albright hereditary osteodystrophy, and pseudohypoparathyroidism type 1B (see Chapter 35).[32]

4.2 Cancer

Aberrant DNA methylation has been observed in various cancers that can lead to global alterations in gene expression. DNA hypomethylated regions in the genome are prone to chromosomal translocations and genome instability that can lead to cancer.[47] Focal DNA methylation changes can result in activation of oncogenes (DNA hypomethylation) or inactivation of tumor suppressor genes (DNA hypermethylation). Mutations in *DNMT3A* are frequently found in acute myeloid leukemia and peripheral T-cell lymphomas.[48–50] Loss of ATRX, the DNA translocase component of the ATRX-DAXX-H3.3 pathway, was described as a cause of α-thalassemia mental retardation on the X (ATRX) syndrome leading to frequent nucleosome loss at CpG islands at the α-globin gene promoter.[51] Defects in DAXX and ATRX are also relevant in various tumors. Exome sequencing of human pancreatic neuroendocrine tumors (PanNETs) has revealed that ~40% of such tumors possess loss-of-function mutations in either *DAXX* or *ATRX*.[52]

Aberrant histone modifications that contribute to carcinogenesis have been observed in various cancers and may serve as diagnostic tools to investigate tumor progression and malignancy.[53] This can result from the process of tumorigenesis or from mutations of factors that comprise the epigenetic machinery. Among such mutations are translocations of the *MLL* genes in acute myeloid leukemia that lead to loss of the SET domain (KMT activity) in the MLL proteins, and recurrent somatic mutations of the *UTX* (*KDM6A*) and *EZH2* genes in various tumor types.[54–56] Mutated histones associated with cancer have been named "oncohistones." For example, missense mutations in the variant histone H3.3 (H3.3K27M or G34R/V) occur in pediatric glioblastomas, and H3.3K36M in pediatric chondroblastomas.[57–59] The "K-to-M" mutant histones can prevent histone methylation by HMTs, such as EZH2 (which methylates H3K27) and SETD2 (which methylates H3K36). Reduction in repressive H3K27 methylation and inhibition of PRC2 activity leads to gain of active H3K4 methylation which promotes the cancer state by blocking differentiation of a neural stem cell causing uncontrolled growth and tumors (pediatric glioblastoma).[57,58] Reduction in active H3K36 methylation leads to a genome-wide gain in repressive H3K27 methylation and redistribution of PRC1 causing derepression of its target genes that block mesenchymal differentiation and accumulation of immature chondroblasts (pediatric chondroblastoma).[60]

4.3 Other Diseases

Defects in epigenetic control mechanisms (mutations, improper deposition, removal, or misreading of epigenetic marks) are not only restricted to imprinting disorders and cancer, but also have been shown to underlie other human diseases, such as cardiopulmonary diseases, diabetes, neuropsychiatric disorders, inflammation, autoimmune diseases, and developmental abnormalities.[32] Studies in model organisms and human tissues have shown epigenetic changes that correlate with aging.[61] The impact of epigenetics on aging in turn can impinge on age-related diseases, such as diabetes, cardiovascular diseases, neurodegenerative diseases, and cancer.

4.4 Epigenetic Therapy

The reversible nature of epigenetic modifications highlights the possibility of epigenetic therapy for various disease states. Comprehensive characterization of genome-wide epigenetic marks has been informative to reveal the status of the epigenome in various diseases, particularly cancers. Targeting the disease-associated epigenome holds promise irrespective of whether it is the cause or consequence of the disease process. Some of the therapies that are under investigation include blocking the enzymatic activity of epigenetic regulators, disrupting crucial protein–protein interactions in the protein complexes that control epigenetic processes, and targeting relevant proteins for degradation.[62] Such therapeutic options could ultimately help to reset the abnormal epigenetic profiles of the diseased state back to normal. Small molecule chemical inhibitors of epigenetic enzymes have been developed (e.g., DNMTi, HDACi, KTMi, KDMi, and BETi). Currently, the two types of epigenetic therapies approved by the US Food and Drug Administration are HDACi (panobinostat, vorinostat, and romidepsin), and DNA hypomethylating agents (decitabine and azacitidine that target DNMT1 and 3A).[63] They have been used in the treatment of specific hematological malignancies. Other classes of drugs under development and/or in clinical trials are those that target-specific interactions in chromatin modification protein complexes or specific epigenetic modifications.[62] Current challenges of epigenetic therapy include cellular heterogeneity of the affected tissue and disease cell-specific targeting, side effects, resistance, and constant, consistent and long-lasting inhibition of the target.

5 CONCLUSIONS

Epigenetic control mechanisms have been grouped into three broad classes, such as posttranslational histone modifications and chromatin remodeling, DNA methylation, and ncRNA interactions. The interplay of these mechanisms in intra- and internucleosomal interactions over short and long distances generate a variety of chromatin states. The sum of these mechanisms is fundamental to the regulation of diverse cellular processes through differential transcriptional readout of the same genetic material. The importance of epigenetics is underscored by many diseases that can develop due to mutations in epigenetic regulatory proteins, misregulation of the epigenetic machinery, and aberrant placement or removal of epigenetic marks. The reversible nature of epigenetic alterations is an attractive target for therapeutics that can help reset the epigenome to the normal state. The fact that some of these epigenetic drugs have been efficacious in the treatment of cancers, such as hematological malignancies reinforces the importance of epigenetics. The recent developments in high throughput sequencing techniques have enabled epigenome profiling of various cell types in their normal or pathological states. These epigenome signatures can be valuable for disease diagnosis, prognosis, and treatment opportunities. Ongoing and future research in the field hopes to shed light on epigenetic changes from a host of inputs, such as aging, metabolic, nutritional, physiological states, environmental conditions, early and late life exposures, chemical, and immunological challenges.

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CHAPTER

3

Genome-Wide Association Studies

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1 INTRODUCTION

In 1989, Lander and Botstein suggested and developed approaches for using markers (initially restriction fragment length polymorphisms and subsequently microsatellites) in multicase families to map quantitative trait loci by linkage.[1] Over the next 15 years, hundreds of genome-wide linkage studies were performed for both dichotomous and quantitative trait diseases, with very little success. While these approaches were adequately powered to identify major gene effects, such as the contributions of HLA loci in immunological diseases, for most common diseases they lacked adequate power to identify disease susceptibility loci robustly. Even if such loci were established, linkage approaches lacked sufficient resolution to pinpoint the responsible genetic variants underneath the linkage peaks. This was certainly true for studies in osteoporosis.[2]

Candidate gene association studies were another widely employed approach that were similarly unproductive. As we do not plan to review these in detail in the current chapter, no candidate gene association reported prior to the genome-wide association studies (GWAS) era in genetic studies of bone mineral density (BMD) variation has been confirmed as a definite osteoporosis gene, since the introduction of robust GWAS design with the single exception of *LRP5*.[3–5]

The development of the GWAS approach and its application over the past decade has completely revolutionized common disease gene mapping. In contrast to previous studies, standard design and analysis approaches in GWAS have led to far more robust and reproducible results. These features include: better quality control of genotypes and cohorts, including identification of cryptic relatedness among the cohort, identification and control of population stratification, and generally more stringent exclusion of poorly performing samples or markers. Further, the sample sizes in GWAS are much bigger than typical candidate gene studies. Considerably more stringent statistical thresholds are used for claiming identification of positive findings, with genome-wide significance set at $P < 5 \times 10^{-8}$. Thereby increasing both the prior and posterior probabilities of a positive finding being a true result, the findings of GWAS have proven much more robust than previous candidate gene studies.

Since the introduction of GWAS for common disease gene mapping, over 10,000 robust genome-wide significant associations have been reported, an extraordinarily rich return (cataloged results at http://www.ebi.ac.uk/gwas/home).[6] Several GWAS in osteoporosis have now been reported,[7–22] and large metaanalyses have now reported 190 genome-wide significant associations, although many of these are replication findings, and the true number of independent associations is smaller than this (NHGRI-EBI Catalog of published GWAS, accessed 14/04/2016). These findings nonetheless only contribute to a small fraction of the overall heritability of osteoporosis phenotypes; and thus far some major traits of interest, such as fracture, have proven harder to dissect using the GWAS approach. The assumptions underlying GWAS, the methodology involved, the strengths and weaknesses of the approach, and likely future developments will be discussed later.

2 LINKAGE DISEQUILIBRIUM MAPPING

In 1996, Risch and Merikangas recognized the limitations of linkage approaches to map genes determining common diseases, and instead proposed that wide-scale association studies using unrelated cases and controls could detect those genes, each of relatively modest effect, underlying most complex common human diseases.[23] At the time, the number of genes in the human genome was unknown, as was the extent of variability and linkage

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disequilibrium (LD) in the genome. However, Risch and Merikangas suggested that testing a million alleles for association in cohorts of achievable size (<1000 individuals) would be an efficient and powerful way to detect genes with modest effects (genotypic relative risk of 1.5).

Risch and Merikangas recommended that the threshold for declaring significance should be $p = 5 \times 10^{-8}$, corresponding to the same risk of type 1 error as represented by a LOD score of 3 when typing 500 markers, while recognizing that this was conservative given the likelihood of LD between variable alleles. Although this remains a stringent threshold for declaring association, most geneticists still regard significant association as proven only when replicated in an independent cohort.

While this threshold has been widely accepted as representing a "significant" finding in GWAS,[24] using such a fixed threshold has weaknesses. For example, the posterior probability of a true GWAS finding depends on the study power (prior probability); thus studies with lower power are more likely to produce false positive findings for the same given *P*-value as larger and/or more powerful studies.[25] Nonetheless, the majority of findings that achieve genome-wide significance have subsequently been replicated in independent cohorts. It is often argued that this threshold is too stringent for candidate gene/locus association studies, in which far fewer markers are studied than in GWAS. While this may be mathematically true, a high proportion of findings at lower significance values prove to be false positives, and thus candidate gene studies achieving "experiment-wise" significance should be interpreted with some caution, if genome-wide significance is not achieved.

At the time of their publication, the study design proposed by Risch and Merikangas was theoretical, as the full extent of variability across the genome was unknown and an efficient means of capturing that variability was technically impossible. Within a decade, both challenges were met. Major technological advances in fields as diverse as chemistry, optics, and computational processing lead to high-throughput array genotyping; and large collaborative genetic programs, such as the International HapMap and Human Genome Project characterized the extent of variability in the genome.[26] This variability includes microsatellites, copy number variants (CNV), and structural variants including insertion/deletions; but the greatest source of variability is single-nucleotide polymorphisms (SNPs). Currently, the number of known SNPs with a minor allele frequency (MAF) >1% is 30 million (source: dbSNP 132). As there are 3 billion bases in the human genome, this represents a SNP every 100 base pairs on average, although variability is not uniform throughout the genome. The number of SNPs being added to public databases is increasing rapidly, and therefore this is an underestimate of the true extent of variation in the human genome.

LD refers to the coinheritance of alleles at two (or more) loci more often than would be expected by chance. To illustrate how LD arises across the genome, a new mutation will arise on a particular ancestral haplotype; the mutation will then be inherited together with the surrounding background stretch of DNA. Thus, unless a meiotic event occurs to separate them, SNPs that are close to the new mutation on the ancestral chromosomal strand will all be inherited together along with the new mutation. At a population level, LD across the genome has decayed through multiple meiotic events such that LD extents only a short distance, typically estimated at approximately 10–20 kB depending on the age of the population.

Through LD, it is possible to build haplotypes or stretches of DNA with a series of correlated markers (such as SNPs); only one SNP needs to be genotyped to allow the genotype of all the other SNPs on the haplotype to be inferred. The International HapMap project was established to characterize the haplotypic structure of the genome [initially for just 4 populations (white Europeans, Chinese, Japanese, and Yoruban Nigerians) but now including 11 other global ancestry groups]. Such data inform and enable such genotyping projects.[26] The earliest commercial SNP chips could not be said to truly capture the whole genome; but with SNP choices informed by LD coupled with technological advances in the number of genotypable SNPs, current commercial whole-genome SNP chips can type up to 5,000,000 SNPs. Further, imputation—by which genotypes at loci not directly genotyped are predicted based on findings from nearby directly assayed genotypes and known haplotypic findings across the locus—can be used to increase the number of genotyped SNPs several-fold above the number directly genotyped.[27]

From a disease-mapping point of view, then, it became possible to look for association across all genes in the genome simultaneously, without bias or assumptions of biological activity. Although the true disease-associated (disease causing) SNP at a particular locus might not be directly genotyped, LD between the disease-causing SNP and the genotyped SNP would mean that association would be detected at the locus, though further fine mapping would be necessary to determine the exact disease-causing polymorphism driving the observed association at this site.

This argument assumes that common diseases are caused by common variants in the population that have arisen in a common founder to whom carriers of the disease-associated variant are distantly related, the so-called "common disease–common variant" hypothesis. To illustrate how this might occur, at some point in the past an ancestor developed a genetic mutation that resulted in a disease. If all current individuals with this disease have arisen from this common founder, then all

cases will share the haplotype carrying the mutation. In contrast, the rest of the population will not carry the disease-associated haplotype. Association with the disease-causing haplotype will be evident even in relatively modest-sized cohorts, depending on the relative risk of disease conferred by the mutation and the number of genes involved in determining disease.

The publication of the Wellcome Trust Case-Control Consortium studies in 2007 illustrated that at least for some diseases the common disease–common variant hypothesis held true.[28,29] Since 2007, hundreds of GWAS have been reported; an up-to-date summary of all published associations at genome-wide significance can be found at http://www.ebi.ac.uk/gwas/home.

In recent times, though, there has been much discussion of the relative contribution of rare variants of greater individual effect size upon a trait than common variants. In one sense this is not surprising: for common variants to become common they cannot exert a major negative effect upon a phenotype or natural selection would result in their removal from the population. How much of the observed association with common variants and disease is due to rare versus common variants remains conjectural; this is discussed in depth later.

3 STUDY DESIGN ISSUES IN GENOME-WIDE ASSOCIATION STUDIES

In GWAS the search is for variants with generally modest effects in a very large experimental space. Thus very careful quality control is required to reduce potential sources of error that could easily translate into false-positive findings. The key aspects of this quality control process are described later.

3.1 Quality Control

Spurious association can arise from SNP misgenotyping, especially if this is different between cases and controls. SNPs that genotyped poorly should be excluded from analysis because of this potential bias, and most studies would exclude SNPs that are genotyped in <90% of the population. Further, individuals who genotyped poorly should also be excluded from analysis as they have a higher proportion of incorrectly called genotypes. Typically a call-rate (proportion of SNPs successfully genotyped in an individual) threshold of 95%–98% per individual is applied.

Individual SNPs should be assessed for deviation from Hardy–Weinberg equilibrium; significant deviation implies unreliable genotyping and the genotyping should be reviewed. This deviation may also occur due to strong, true association, so thresholds tend to be liberal here ($p < 10^{-7}$ being a common threshold).

Per subject heterozygosity is also used as a filter. Excess heterozygosity may indicate DNA mixing/contamination, and low heterozygosity may indicate either low-quality genotyping or population stratification.

Identification and exclusion of related individuals is critical in GWAS studies as the statistical analysis assumes that subjects are unrelated. It is quite common for relatives to be recruited accidentally into studies, and even for the same individuals to be rerecruited. Screening for identical names, date of birth, and gender before genotyping can identify some duplicate individuals; however, this approach cannot identify siblings or other types of relative pairs. This can be achieved genetically by measuring the extent of sharing of SNP genotypes between all pairs of individuals in the study. Related individuals have excess sharing of SNP genotypes; this approach can be used reliably to identify and exclude individuals who are up to third-generation relatives (cryptic relatedness).

The robustness of association depends critically on an overall common genetic background between cases and controls. If these subjects are drawn from different ethnic groups, there will be differential SNP sharing simply due to their different ethnic background unrelated to the presence or absence of disease; this is referred to as population stratification and is a potent cause of inflation of GWAS findings. Investigators should make concerted efforts to minimize ethnic heterogeneity in their study subjects, but some diversity is inevitable. This is particularly the case where a common control data set is used, as the controls are not necessarily recruited from the same geographic region as the cases. To address this, principal components analysis methods have been developed that identify groups of SNPs that distinguish strata of the population from one another.[30] This is a powerful method for distinguishing even minor differences in ethnic background between subjects in GWAS studies (Fig. 3.1). Typically, subjects who are ethnically remote to the main cohort are excluded from further analysis. Weighted scores of ethnicity defined by principal components analysis or related methods can then be used to control for minor differences in ethnicity between the remaining subjects.

Another method of assessing population stratification is to compare the number of observations to the number expected by chance (presented as a quantile–quantile plot). Significant deviation implies that there are differences between the cases and controls not simply due to differences in disease prevalence. The extent of this deviation is assessed using the mean or median association strength statistic compared with the null hypothesis across all SNPs studied (referred to as the genomic inflation factor[32]). This factor can be inflated by error (such as cryptic relatedness of samples, genotyping error, or population stratification), or by true association. A genomic

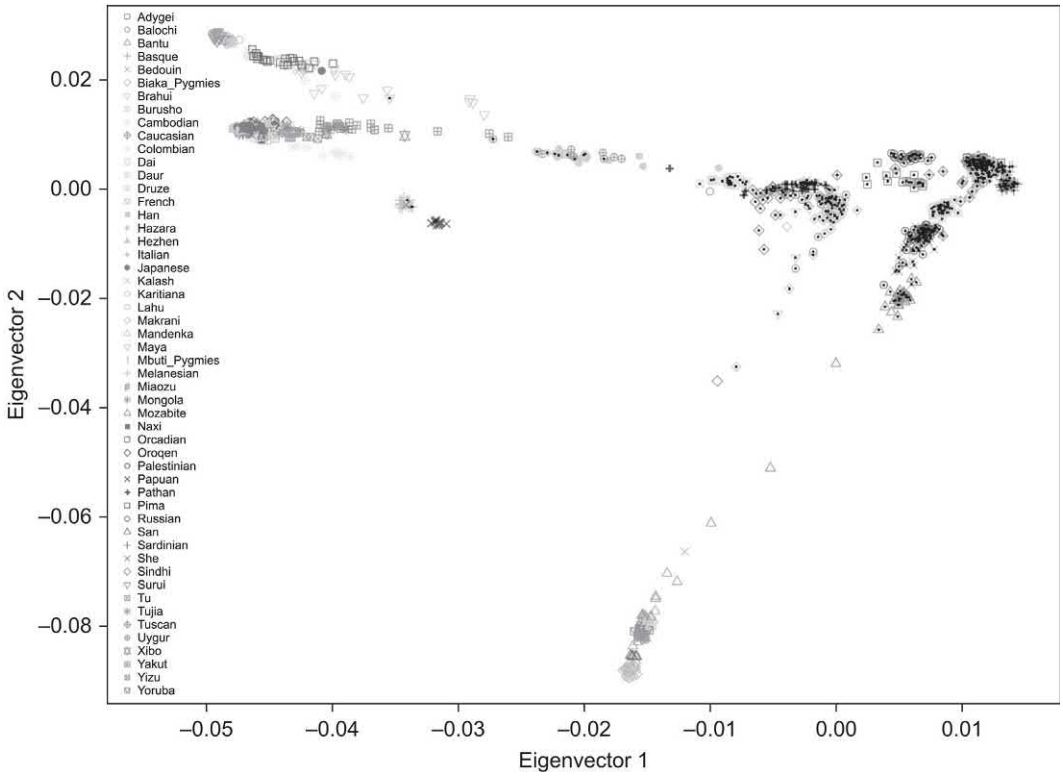

FIGURE 3.1 Principal components analysis ancestry assessment. Forty-nine ancestry-informative markers were used to distinguish the ethnicity of 1104 samples from the Illumina iControl database, reported to be from 52 ethnic groups. Those marked with a black dot were confirmed not to be of white European ancestry.[31] Those with African or Asian ancestry were easily distinguished; and ancestry was able to be determined for even quite closely related ethnic groups.

inflation factor of 1.0 indicates no evidence of inflation of association findings from the null (and in fact if truly exactly 1.0 would represent a negative study), and values up to 1.10 are generally considered acceptable for GWAS studies. The genomic inflation factor increases with sample size; to provide a measure comparable across sample sizes, corrected statistics for a sample size of 1000 subjects are usually also reported.

The genomic inflation factor can be used to correct average inflation of test statistics across the genome, but this approach assumes that the test statistics are equally inflated at each locus across the genome, which is not usually the case. Rather, stratification tends to affect loci that are under selection pressure, such as genes involved in immunological and nutritional pathways.[28] Therefore other quality control measures are essential to provide robust results. The gene encoding lactase (*LCT*), of obvious potential importance in osteoporosis, is highly subject to population stratification; this is likely to account for early studies reporting its association with BMD. *LCT* has not been identified as a susceptibility locus in osteoporosis GWAS, probably because GWAS can properly control population stratification effects.

4 THE "MISSING HERITABILITY" QUESTION

Despite ever larger and more powerful GWAS and metaanalyses, the proportion of genetic variance explained in different common diseases has, with few exceptions, remained low. This is also the case in osteoporosis and related phenotypes, such as height. One of the largest studies yet performed concerning osteoporosis is the second Genetic Factors in Osteoporosis study, which in its discovery arm involves 32,961 individuals, and in its replication arm 50,933 subjects. This study identified and replicated 56 loci associated with BMD, substantially increasing understanding of the genetic architecture of BMD variation. However, a total of just 5.6% of BMD variation was explained, compared with the heritability of BMD of over 60%.[17] In the fracture component of this study, 31,016 cases and 102,444 controls were studied, identifying 14 SNPs associated with fracture (at "experiment-wide" significance, in this case $p < 5 \times 10^{-4}$), explaining just 2.3% of fracture risk.

The failure of GWAS to identify larger proportions of the variance of traits has led to much discussion as to why this might be the case, and consideration of alternate

study designs to capture more of the variance.[33] Potential repositories of unmapped genetic variance include rare variants, CNVs, insertion/deletions, and epigenetic factors, such as methylation.

The number of common variants identified in GWAS scales are almost linear as the sample size increases, almost independent of the heritability of the disease or trait studied,[34,35] and thus it is possible that simply by further increasing sample sizes, a higher proportion of the variance will be explained. Against this, it is hard to imagine that GWAS of hundreds of thousands of subjects will be possible for most traits or diseases. Further, with ever larger GWAS, the effect size of new SNPs being identified diminishes. For example, in Crohn's disease, the initial 30 loci explained 20% of the variance, and the next 39 loci only added a further 3.2% of the variance explained.[36] This diminishing return with ever larger datasets indicates that while increasing the sample size will be helpful, other variants not captured by GWAS chips must exist.

GWAS chips are designed to optimally tag common variants across the genome. Chip manufacture companies, such as Illumina (San Diego, California) and Affymetrix (Santa Clara, California) have adapted their products to improve capture of CNVs and rare variants. There is now good evidence that common CNVs that can be typed by our best available technology do not contribute significantly to missing heritability, and indeed have largely been tagged by SNPs on chips already. In a large Wellcome Trust Case-Control Consortium study of CNVs in eight different diseases, it was demonstrated that any common CNV associations observed had already been identified through SNP marker studies.[37] It remains possible that rare CNVs or those too small to be detected by SNP chips (such as insertion–deletion polymorphisms, termed "indels") may contribute to missing heritability, but it will require large studies using improved genotyping technology to address these.

The relative contribution of rare and common variants to common disease heritability is unclear currently. Research using the model trait of height provides insights as to the likely repositories of unmapped genetic variants. Considering GWAS data from height studies, it has been demonstrated that 45% of the narrow-sense heritability (due to additive effects of individual loci, without interaction between then) of height is due to common variants. At this point genetic associations that determine 5% of the variation in height have been identified, leaving 45% − 5% = 40% of total height variation to be due to common variants that have yet to be pinpointed. Thus 40%/45% or 88% of genetic variation in height is due to variants that have yet to be pinpointed. As the heritability of height at the time of this analysis was thought to be ~80%, 80% − 45% = 35% of the heritability of height is due to genetic factors other than common variants captured by GWAS.[38]

The ability to detect rare variants using imputation has substantially improved with increasingly large reference haplotype datasets. Using the 1000 Genomes project reference haplotype data for imputation, with genotyping performed using a standard GWAS chip (the Illumina CoreExome chip), 96% of common genetic variation and 73% of rare variation detected in the UK10K whole genome sequencing dataset could be identified. The heritability of height estimated from pedigree studies is thought to be not greater than 69% (and may be lower than this). Heritability estimates from imputed data in 44,126 individuals estimated common variant heritability captured by the GWAS-imputation approach as 56%. This is close to the likely overall heritability.[39] Thus missing variation in height is negligible at this sample size. This study also confirmed that rarer variants contribute more in total to height variance than more common variants, as well as individually having larger effects in carriers; there is some support that this is also the case in BMD variation.[22] It is very likely that these findings can be extrapolated to BMD and osteoporosis risk, and that very large-scale GWAS will be required to capture sufficient genetic variation to enable more accurate genetic risk prediction in osteoporosis.

Skeletal phenotypes are those areas where there is clear overlap between rare and common variant associations. For example, in a GWAS using a unique extreme-ascertainment design, we recently demonstrated association of BMD with common variants in six novel loci (*GALNT3*, *RSPO3*, *CLCN7*, *IBSP*, *LTBP3*, and *SOX4*), and replicated 21 of the then 26 known BMD loci.[40] Of the novel associations, rare variants in *GALNT3*, *CLCN7*, and *LTBP3* were already known to influence BMD. Thus, while BMD may be largely determined by common variants, there is strong a priori evidence that rare variants are also associated with osteoporosis, validating further studies to identify these.

Although gene–gene interaction has been demonstrated in many model organisms,[41,42] it does not contribute to narrow-sense heritability, as assessed in twin studies, and therefore does not explain missing heritability. Few examples of replicated established gene–gene interaction exist for any common human diseases, the most established involving variants of the endoplasmic reticulum aminopeptidase gene *ERAP1* and HLA Class I loci, in ankylosing spondylitis[43] and psoriasis,[44] although epistasis affecting gene-expression appears much more commonly.[45] Gene–gene interaction may be widespread, but current studies are underpowered to detect such effects.

Methylation studies in common human diseases are in their infancy. Affordable, high-throughput, and robust methods for genome-wide analysis of methylation

status have only recently been developed. While more comprehensive whole genome assessment of methylation status by sequencing is possible, such methods are not yet suitable for large-scale studies due to cost and complexity issues. The heritability of DNA methylation has been shown to be significant (0.199 average in peripheral blood, corrected to 0.176 once cellular composition of blood is accounted for) though this figure is somewhat lower than most diseases. Thus methylation varies both from modestly heritable transgenerational effects and from environmental exposures (as methylation is one mechanism by which the genome responds to the environment, reviewed in Ref. 46). Methylation varies between tissues, as well as at different time points in the same individual, and it is not yet clear to what extent methylation patterns in accessible tissues, such as peripheral blood reflect patterns in nonaccessible tissue, such as bone, obviously of critical importance to such studies in osteoporosis. Methylation studies not only require tight control for major environmental covariates, particularly smoking but also age, to avoid spurious results. Nonetheless, it is possible given the heritability of methylation that it too may contribute to unmapped heritability.

5 RARE VARIANT STUDY DESIGNS

Rare variant studies pose significantly greater challenges for gene-mapping studies to common variant studies. These include sample size requirements, genotyping issues, and analysis approaches.

Power for gene-mapping studies depends on the sample size, extent of LD between the genotyped and disease-causing variants, the allele frequencies of those variants and effect size of the disease-causing variant. For variants with a given odds ratio for disease, less-frequent variants have a lower effect size in the population. Thus rare variant studies require much larger cohort sizes than are required for identification of common variants. Sample sizes for GWAS of a quantitative trait (such as BMD) and a dichotomous trait (fracture) are illustrated in Fig. 3.2 shows that rare variant studies require very large sample sizes unless the individual effect size of the rare variants are large, and much larger than that observed for common variants to date. For example, a study of 10,000 fracture cases and 10,000 no-fracture controls would have 80% power to detect a SNP with MAF of 0.1 with a heterozygote odds ratio of 1.2; but for a rare variant SNP with MAF of 0.01, the same study would only have 80% power if the SNP were associated with fracture risk with a heterozygote odds ratio of 1.63, which odds ratio would generally be considered quite high for a common disease. These theoretical estimates are supported by the experience in real-world

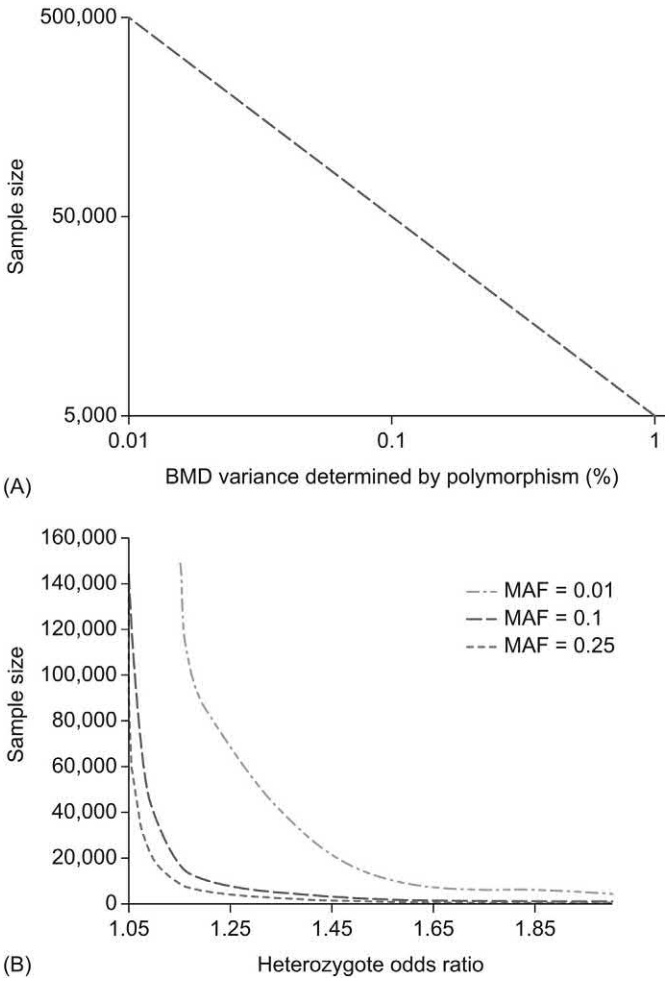

FIGURE 3.2 Sample size requirements for genome-wide association studies (GWAS). Model assumes linkage disequilibrium (LD) (D′) between marker and disease-causative variant of 0.9, equal marker and disease-causative variant allele frequencies, power = 0.8, and $\alpha = 5 \times 10^{-8}$. (A) Sample size for the quantitative trait bone mineral density. (B) Sample size for fracture studies, assuming fracture risk of 20% and an equal number of cases and controls. *MAF*, Minor allele frequency.

gene-mapping studies. For example, a recent study involving 2882 whole genome sequenced subjects, 3549 whole exome sequenced subjects, and 26,534 GWAS genotype and imputed subjects identified one additional novel locus associated through a low frequency but not rare variant (*EN1*, rs11692564, MAF 1.7%).[22] This high-quality study illustrates how hard identifying true rare variants is going to be without much larger sample sizes.

Again considering height as a model trait, Fig. 3.3 shows the proportion of variation captured by height GWAS studies plotted against sample size. Assuming that the genetics of height and BMD variation are similar, it can be extrapolated from Fig. 3.3 that studies of the order of 500,000 samples will be required to capture >90% of BMD variance at genome-wide significance. Such

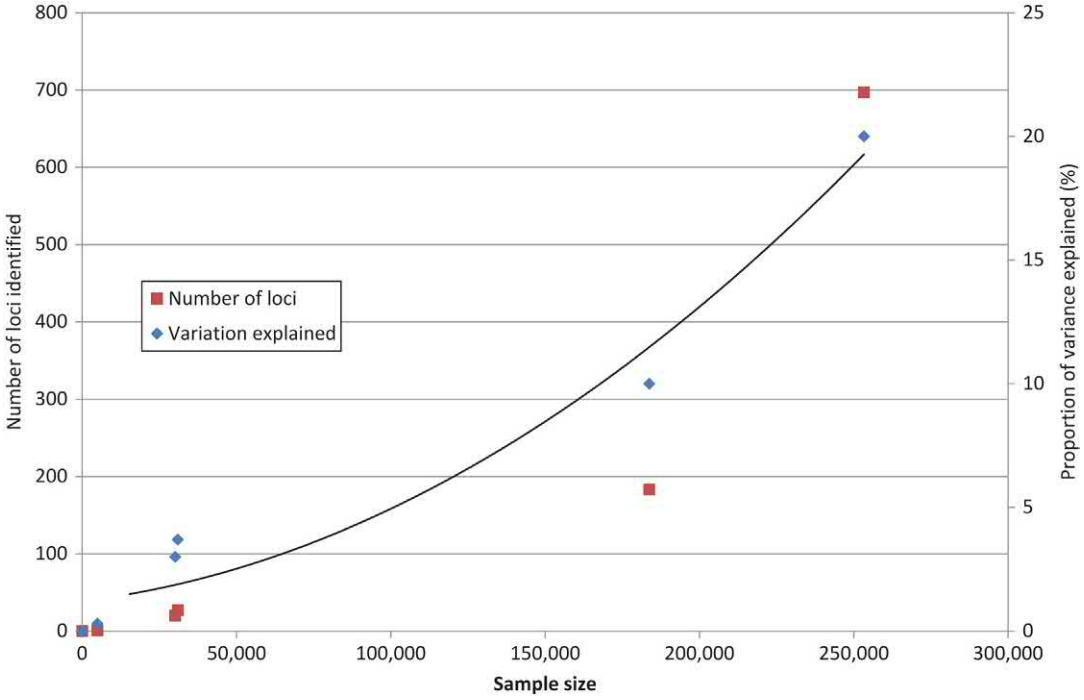

FIGURE 3.3 Genetic studies of height, comparing sample size with number of loci identified and proportion of variance explained. A polynomial trend line has been fitted to the proportion of variance data (r2 = 0.97).

studies [e.g., the UK Biobank study (http://www.ukbiobank.ac.uk/)], are currently in progress, and should provide a definitive view of common and rare genetic variation in BMD. Similarly, large-scale cohort studies will be required to capture a high fraction of variation in fracture risk due to both rare and common variants. Sequencing studies (either whole exome or genome) in either families or cohorts of unrelated individuals with extreme bone phenotypes are likely to be more efficient at identifying disease-associated rare variants or mutations than general population cohort studies. Gene mapping using this approach has been highly successful for extreme bone phenotypes.[47,48] Early studies have shown that studying cases or small families with extreme bone density shows an overrepresentation of mutations in genes known to be involved in high and low bone density diseases.[49] This suggests that more comprehensive genetic screening of similar cohorts would an efficient method for discovering further bone density associated genes.

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The rapid improvement in the quality and accuracy of imputation allowing in silico genotyping means that this approach is currently the preferred option for capturing rare genetic variant associations. As the known number of rare variants in public databases is extremely large, and is still increasing, existing publically available reference haplotype sets can be used for high-quality imputation of both rare and common variants in already GWAS-genotyped samples. This is a much more cost-effective means of studying rare variants than adopting sequencing approaches wholesale. Whole exome and whole genome sequencing are becoming cheaper—but so are genotyping chips, currently about 100× cheaper than a whole genome scan. Given the large sample sizes needed for adequately powered rare variant studies, the additional cost of sequencing makes this approach currently unaffordable. As many reference imputation datasets are based on healthy controls, they may not contain low-frequency risk or protective variants for diseases, and thus study designs involving sequencing of extreme BMD cases or low trauma fracture cohorts to increase the capture of relevant genetic variants in the reference imputation datasets seems a logical and more cost-effective alternative study design.

6 CONCLUSIONS

GWAS has become the tool of choice for hypothesis-free investigation of the causes of common heritable diseases. With careful quality control and appropriate conservative interpretation, this approach has been very successful in identifying thousands of genetic loci involved in human diseases. Imputation methods now mean that nearly all common genetic variation, including the majority of rare genetic variation, can be captured by low cost microarray SNP GWAS studies followed by imputation, rather than whole genome sequencing. Very large-scale (~500,000) subject studies are going to be required to capture the high proportion (>90%) of genetic variance required for genetic risk prediction to become clinically useful. Such studies are currently underway, making it likely that within the next couple of years the genetic architecture of osteoporosis will be largely characterized. The priority has now shifted from gene mapping to translating these advances into clinically relevant contributions, such as the development of novel therapies for osteoporosis.

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CHAPTER

4

Copy Number Variation

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1 INTRODUCTION

Humans manifest substantial phenotypic heterogeneity [e.g., height, body mass index (BMI), hair color, eye color, skin color, and susceptibility to diseases]. Nevertheless, one striking conclusion from The Human Genome Project is the similarity of DNA sequences among different individuals; approximately 99.9% of the DNA sequence from any two unrelated humans is identical.[1–3] Therefore, the extent to which phenotypic heterogeneity can be explained by differences in DNA sequence seems attributable only to relatively minor variations. Consequently, single-nucleotide polymorphisms (SNPs) have become the focus of a large number of studies designed to identify critical differences in DNA sequence, which contribute to phenotypic variation for specific traits.[4] Although this approach has been highly productive, resulting in the identification of numerous associations between novel SNPs and a range of common and complex diseases, only a modest degree of phenotypic variation has been explained by SNPs.[5–7] This has led to broader hypotheses regarding the potential genetic basis of this "missing heritability."[6]

Copy number variation (CNV) has been proposed as one other type of genetic variation that contributes to phenotypic variance.[8] For nearly all genes in the human genome, we inherit one copy from each parent, and thereby have two copies in the nucleus of every diploid cell. For some genes, however, the copy number varies from this norm. For example, several β-defensin genes represent examples of common CNVs, with between two and seven copies per diploid cell.[9] Another common example of a CNV involves the UDP glucuronosyltransferase 2 family, polypeptide B17 gene, *UGT2B17*. East Asian populations often have a homozygous deletion of this gene resulting in zero copies in diploid cells.[10,11] CNVs, then, are defined as structural variations in chromosomes representing DNA segments of 1 kb or more that are present at varying copy numbers in the genome of different individuals.[8,12] CNV may either be inherited from a parental genome, or caused by mutation designated de novo CNV. In this case, the region of de novo CNV in both parent's genome is expected to be normal. CNVs may manifest as simple deletions, insertions, or duplications (Fig. 4.1). A CNV that reaches a population frequency greater than 1% is defined as a common CNV, or as a copy number polymorphism (CNP). CNVs that are found in less than 1% of the population, in contrast, are referred to as rare CNVs.

The first discovery of CNV in humans can be traced back to the early 1900s when a man was identified with a single X chromosome and no Y chromosome.[13] Trisomy 21 represents the first discovery of human autosomal CNV. Identification of these CNVs relied on relatively insensitive techniques that only permitted detection of variations that could be detected using optical microscopes, such as aneuploidies, marker chromosomes, gross rearrangements, and variations in chromosome size.[14,15] The population frequency of these early discoveries of gross CNVs, however, was very low and was directly related to specific genomic disorders.[16–18] Accordingly, the contribution of smaller CNV toward more subtle phenotypic variations was not yet generally appreciated. Now, we recognize that most CNVs or CNPs represent submicroscopic chromosomal structural variations that escape detection with even the most powerful optical microscopes. A major contribution to our understanding of these submicroscopic CNVs was attributable to Redon et al.[8] who constructed the first comprehensive

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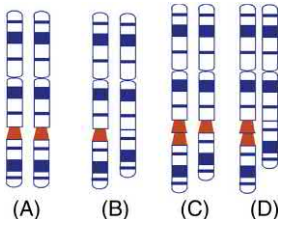

FIGURE 4.1 Copy number variation (CNV). The *trapezoid* delineates the region with variation. (A) Normal copy numbers; (B) deletion; (C) duplication; and (D) multiallelic loci with a duplication on one chromosome and a deletion on the other chromosome.

human CNV map, using 270 HapMap individuals,[8] and observed that CNVs exist in healthy people. Among their 270 study subjects, almost 12% on an average of the whole genome in each individual, approximately 360 Mb, had CNVs. Most of these CNVs were of relatively small size (less than 20 kb), and many were present at >1% in this population, thus being categorized as polymorphisms (CNPs). With the increasing number of published high-quality data, Zarrei et al.[19] constructed an updated CNV map of the human genome recently. They estimated that 4.8%–9.5% of the genome contributes to CNV, which is lower than that of what Redon et al. reported. In the meantime, they found approximately 100 genes that can be completely deleted without producing any apparent phenotypic consequences.[19] Likewise, Shen et al. also observed general gene "knocked out" and provided a comprehensive characterization of human genomic variation as well, by performing a whole genome sequencing study in 44 unrelated healthy Caucasian adults.[20] As it became increasingly apparent that there are interpopulation differences in gene copy numbers,[8,21] it was hypothesized that CNVs could be comparable to SNPs in playing important roles in adaptation to different environments, susceptibility to common diseases, and evolution. However, if SNPs can serve as proxies for CNVs,[22,23] it is not necessary to investigate CNVs, as the methods of SNP genotyping and data analysis are much easier than those of CNVs. To validate this hypothesis, Stranger et al.[24] studied the relative contribution of CNVs and SNPs to variations in gene expression in lymphoblastoid cell lines from 210 unrelated HapMap individuals. They found that SNPs and CNVs captured about 80 and 20%, respectively, of the detected variation in gene expression. Importantly, there was little overlap in the signals from these two sources of variation.[24] This finding strongly supports the conclusion that CNVs critically regulate gene expression.

1.1 Potential Mechanisms for Formation of CNVs

CNVs often occur in regions that contain segmental duplications.[25–28] Segmental duplications are blocks of DNA that typically share more than 90% sequence identity and occur at more than one site within the genome.[29] The well-known mechanism for CNV that is associated with segmental duplications are nonallelic homologous recombination mechanisms (NAHR).[30] Homologous recombination is thought to be a classical mechanism for promoting genetic diversity.[31] For instance, in a meiotic diploid cell, recombination can switch allele combinations along a linear chromosome. Therefore, a novel haploid in the subsequent generation is produced. NAHR is a form of homologous recombination that occurs between two lengths of DNA that have high sequence homology, but are not alleles.[32] When NAHR occurs between sequences of DNA that have different low-copy repeats, copy number changes (deletions or duplications) of the DNA can occur.[33,34] For example, in a diploid cell, each allele has two copies at a specific chromosome site. There is no deletion or duplication in this generation. However, when NAHR occurs, there is an exchange between the two copies from one allele and one copy from another allele, thereby producing a novel allele (one copy or three copies) for the subsequent generation.

Not all CNVs are associated with segmental duplications. Some CNVs are thought to arise via another recombination-based mechanism, nonhomologous end joining (NHEJ).[35] This is a pathway that repairs double-strand breaks in DNA. In NHEJ, when double strand DNA breaks are detected, the broken DNA ends will be bridged, modified, and then ligated. During this process, the product of repair often contains additional sequence at the DNA end junction, leaving a "DNA scar."[36,37] After NHEJ, for this additional sequence, it might be a duplication in contrast to cells with no NHEJ.

Another formation of nonhomologous junctions linked to DNA replication has been regarded as the replicative mechanism. During replication, the sequence between the homologous regions, expected to be single stranded, is often deleted or duplicated. This deletion or duplication has been attributed to a mechanism of replication slippage along the exposed template during DNA replication.[38,39] There is growing evidence for the involvement of replication in underling copy number change. For example, aphidicolin, an inhibitor of replicative DNA polymerases, induces CNV at chromosomal fragile sites and throughout the genome.[40–42]

2 CNV DETECTION

Several methods have been developed for analysis of unbalanced chromosomal structural variants, we have summarized the most common used methods in Table 4.1. For CNV detection, the common approaches are array-based analyses[8,11,26,27,43–49] and quantitative, primarily polymerase chain reaction (PCR)–based,

TABLE 4.1 Summary of CNV Detection Methods

Schemes	Technologies	Characteristics	Platforms for examples	References
Genome-wide CNV detection	CGH arrays	Reference and test DNA comparing by labeling different fluorescent tags	Roche NimbleGen, Inc.	55
	SNP arrays	SNP genotyping	Affymetrix Genome-Wide Human SNP Array 6.0	11
	Resequencing	High-throughput DNA sequencing (next-generation sequences)	Illumina/Solexa Genome Analyzer	57
Candidate CNV detection	qPCR	CNV candidate regions genotyping by detecting the C_t value		8
	MLPA and MAPH	Simultaneous analysis of multiple genomic regions		50,54

CGH, Comparative genome hybridization; SNP, single-nucleotide polymorphism; qPCR, quantitative real-time polymerase chain reaction.

assays.[8,11,12,50–54] Array-based comparative genome hybridization (array-CGH) approaches[8,55,56] and SNP arrays[8,11] are the most powerful and robust methods for genome-wide CNV detection.

1. *Genome-wide CNV detection*
   - **a.** *Comparative genomic hybridization arrays*: CGH arrays represent the most widely used method for genome-wide CNV identification. This approach uses different fluorescent tags to label normal control (reference) DNA and test DNA. Then, reference and test DNA are simultaneously hybridized to the array, and CNV genotype can be inferred by comparing the respective signal intensities. Bacterial artificial chromosome (BAC) clones, which can cover most of the human genome, serve as the source for standard CGH arrays.[55] Although BAC arrays provide robust coverage of the genome and a high signal-to-noise ratio, the resolution is relatively low; they cannot detect CNVs smaller than 50 kb, due to the large size of BAC probes.[18,58,59]

     As a result of the development of microarray technology, high-density oligonucleotide arrays are available for CNV detection. For example, a 385K human tiling array and a 2.1-million oligonucleotide array are commercially available through Roche NimbleGen, Inc. (Madison, Wisconsin, USA). Compared to BAC arrays, the resolution of these arrays is significantly improved, but the signal-to-noise ratio from hybridization intensities is decreased.[60]
   - **b.** *SNP arrays*: SNPs have received considerable attention as a major source of human variation.[4] The development of high-throughput array technologies for SNP genotyping has made genome-wide association study (GWAS) feasible.[61] Although these arrays were initially designed for simple SNP genotyping, they are increasingly "mined" for CNV analysis by reanalyzing probe intensity information.[8,11] For the Affymetrix GeneChip 500K (Santa Clara, California, USA), although SNPs have a median spacing of 2.5 kb, the resolution of this array is better because it has a lower limit of 10–40 kb due to the uniformity of probe distribution across the genome.[60] Another problem for this array is that the boundary of a CNV can only be determined by the position of the analyzed SNPs, which are unlikely to be present at the exact start or end point of the CNV. Thus, SNP arrays cannot be used to delineate the precise boundaries of each CNV. Arrays available from both Affymetrix and Illumina (San Diego, California, USA) have added specific probes for CNV detection. For example, the Affymetrix Genome-Wide Human SNP Array 6.0 features 1.8 million genetic markers, including more than 906,600 SNPs and an additional 946,000 CNV-specific probes. Thus, the resolution and boundary problems mentioned previously are significantly improved with these latter arrays.
   - **c.** *Resequencing*: The precise sequence of nucleotides in a DNA sample can be determined by DNA sequencing, and analysis of these data can provide highly accurate information about CNVs. For example, by comparing two completed sequence assemblies, Khaja et al.[57] successfully identified 13,066 previously undescribed structural variations in the human genome, including 419 CNVs. Unlike array-based approaches, DNA sequencing provides revolution on understanding the structure and specifying the location. The most important benefit of this technology is that it is possible to discover a multitude of variant classes with a single sequencing experiment.[62]

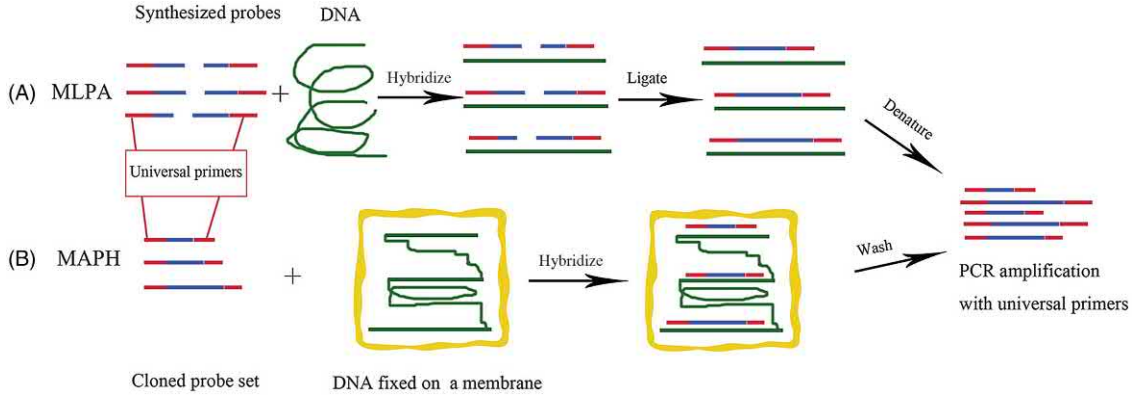

FIGURE 4.2 (A) Multiplex ligation-dependent probe amplification (MLPA) and (B) multiple amplifiable probe hybridization (MAPH).

With the rapid development of high-throughput DNA sequencing ("next-generation" sequences), such as Roche/454FLX, the Illumina/Solexa Genome Analyzer, and the Applied Biosystems SOLiDTM System,[63,64] it is feasible to sequence complete genomes of increasing numbers of subjects and contribute to accurate identification of genomic variations. Based on the next-generation sequencing technologies, Duan et al. practically developed different procedures for CNV detection. For instance, they proposed a method by optimizing a total variation penalized least square criterion,[65] as well as a novel and robust method to detect CNV from short sequencing reads derived from the next-generation sequencing data.[66] As this approach is increasingly utilized, genome comparisons and CNV identification will become more robust and reliable.[67]

2. *Candidate CNV detection*

a. *Quantitative real-time PCR* (*qPCR or RTPCR*): Although quantitative real-time PCR is not suitable for the simultaneous amplification of a large number of targets, it has been one of the most commonly used methods for genotyping candidate regions for CNV.[8] qPCR uses a fluorescent tag to monitor signal intensity for each cycle. The greater the number of copies of a target sequence within a DNA fragment, the lower the C_t value (i.e., the number of cycles required for the fluorescent signal to exceed background levels). Thus, qPCR is an efficient method for detecting deletions or duplications at a single locus. Alternatively, as the developing technology, digital or single-molecular PCR allows screening of samples and sites in an emulsion or a microfluidic device.[68,69] With the benefit of accurate enumeration of PCR results, these technologies allow the detection of events located on a single DNA fragment, which allows analysis of rare subpopulations or individual alleles.[70]

b. *Multiplex ligation-dependent probe amplification* (*MLPA*) *and multiple amplifiable probe hybridization* (*MAPH*): MLPA and MAPH both represent targeted PCR-based methods that simultaneously analyze multiple genomic regions.[50,54,71] MLPA works by quantifying probes that hybridize to genomic DNA and amplified by PCR.[72] The clever design of MLPA is the synthesized probe (Fig. 4.2A). Each probe includes two oligonucleotides, one synthetic "left probe" oligonucleotide and one "right probe" oligonucleotide. Both of them are flanked by a universal primer sequence and are derived from the M13 bacteriophage. When both probe oligonucleotides are hybridized to their respective targets, they are ligated into a complete probe and amplified by PCR with universal primers. The products of the universal primers have unique sizes that can then be separated by capillary electrophoresis. In the MAPH technique (Fig. 4.2B), genomic DNA must be fixed to a membrane, and then hybridized with a set of probes. The probes must be of different sizes, and their products must be distinguishable by electrophoresis. Multiple amplifiable probes are prepared by PCR amplification using common primers flanking the vector-cloning site. Amplification products are analyzed by slab gel or capillary electrophoresis. For both MLPA and MAPH, reduced peaks are indicative of deletions whereas enhanced peaks indicate duplications.

3 CNV AND DISEASE

CNVs, including duplications and deletions, can influence gene expression by disrupting gene coding sequences, perturbing long-range gene regulation, or altering gene dosage. Through these mechanisms, CNVs are believed to cause, or affect susceptibility to, human diseases.[18,37,73] The CNV-related diseases investigated so far are summarized in Table 4.2.

1. *CNV and rare human diseases*: The relatively crude methodology of microscopic chromosomal examination to detect variations in chromosome structure or number has contributed to our recognition of associations between CNVs and human disease.[37] For example, the duplication of chromosome 21 (trisomy 21) results in Down syndrome (DS)[74] and deletion of chromosome 15q11.2-q12 causes Prader–Willi syndrome

TABLE 4.2 Summary of CNV-Related Diseases

	Diseases	Pathomechanisms	References
Rare human diseases	DS	Duplication of chromosome 21 (trisomy 21)	74
	PWS	Deletion of chromosome 15q11.2–q12	75
	Williams–Beuren syndrome	Microdeletion on chromosome 7q11.23	76
	SMA	Deletion of exons 7 and 8 of *SMN* gene	77
	VCFS	Deletion of ~3 Mb in chromosome 22q11	78
	CHARGE syndrome	De novo 4.8-Mb deletion in 8q12	49
	Syndactyly and craniosynostosis	A 9-kb duplicated region located at 40-kb upstream of *IHH*	79
	Monogenic diseases (deletions and duplications involving the X chromosome)	*AR* gene of Xq11-q12 in androgen-insensitivity syndrome	49,80,81
		COL4A5 gene of Xq22.3 in X-linked Alport syndrome	
		IDS gene of Xq28 in mucopolysaccharidosis type II	
		RS1 gene of Xp22.2–p22.1 in X-linked juvenile retinoschisis	
		DMD gene of Xp21.2 in Duchenne muscular dystrophy	
		Insertion on chromosome Xq27.1 in X-linked recessive hypoparathyroidism	82
Common human diseases	Obesity	Deletion near the *NEGR1* gene	83
		CNV of *PPYR1* gene	84
		CNV on chromosome 11q11	85
		CNV on chromosome 15q11–q13	86
	Schizophrenia	CNVs on chromosome 1q21.1 and 15q13.3	87,88
		Deletion on chromosome 17q12	89
		Large duplications (1.5–3.0 Mb) at 22q11.2	90
	Autism	600-kb deletions and duplications on 16p11.2	91
		CNV of *NLGN1* and *ASTN2* genes	92
	Cancer	CNV of *PAX5* gene in ALL	93
		MICA and *HCP5* gene deletions on chromosome 6p21.3 in NPC	94
		Amplifications of *NFE2L2*, *MYC*, *CDK6*, *MDM2*, *BCL2L1*, and *EYS*; deletions of *FOXP1*, *PTEN*, and *NF1* in lung squamous cell carcinoma	95
	CHD	CNV of *NODAL*, *CFC1*, and *NOMO3* genes	96

ALL, Acute lymphoblastic leukemia; CHARGE, a pleiotropic disorder characterized by coloboma, heart defects, choanal atresia, and retarded growth and development; CHD, congenital heart disease; DS, Down syndrome; NPC, nasopharyngeal carcinoma; PWS, Prader–Willi syndrome; SMA, spinal muscular atrophy; VCFS, velo–cardio–facial syndrome.

(PWS).[75] As technology has improved, our ability to determine the contribution of CNVs to human disease has increased dramatically, and CNVs are now known to contribute to cause several additional rare human diseases. In 1993, a microdeletion on chromosome 7q11.23, that included the elastin gene, was found to cause Williams–Beuren syndrome, a disease characterized by mental retardation, infantile hypercalcemia, and a variety of congenital heart defects.[76] In 1997, Campbell et al. [77] found that exons 7 and 8 of the telomeric SMN gene, located on chromosome 5q13, were not detectable in more than 90% of patients with spinal muscular atrophy (SMA). Subsequent examination of point mutations and gene conversions in the *SMN* gene confirmed that CNV played a major causal role in SMA disease. In the same year, Carlson et al. determined that 83% of 151 patients with velo–cardio–facial syndrome (VCFS), a developmental disorder characterized by craniofacial anomalies, conotruncal heart defects, and disturbance in calcium levels, had a deletion of ~3 Mb in chromosome 22q11.[78] This region contains many genes, including *GSCL*, *CTP*, *CLTD*, *HIRA*, and *TMVCF*. Further study of hamster–human somatic hybrid cell lines derived from VCFS patients, showed that the breakpoints occurred within similar low-copy repeats termed LCR22s.[97] Additionally, 18 patients with CHARGE syndrome, a pleiotropic disorder characterized by coloboma, heart defects, choanal atresia, and retarded growth and development, were tested for CNV using a 1-Mb resolution genome-wide BAC array.[49] One de novo 4.8-Mb deletion was identified on 8q12 in a patient. Sequence analysis revealed that the causative mutation involves the *CHD7* gene, a novel member of the chromodomain helicase DNA-binding gene family. Later in 2011, Klopocki et al.[79] identified duplications at the Indian hedgehog (*IHH*) locus, a morphogen of the hedgehog family, in three unrelated families affected by syndactyly and craniosynostosis. These syndromes related to the disorder of bone formation. After describing a 9-kb duplicated region located at 40-kb upstream of *IHH*, which encompassed potential *cis*-regulatory elements, the authors speculated that the duplications interfere with the looping and interaction mechanism of the *IHH* promoter and several *cis*-regulatory elements.

Large deletions and duplications involving the X chromosome have been implicated in a number of serious monogenic diseases. Pertinent examples include the *AR* gene of Xq11–q12 in androgen-insensitivity syndrome, the *COL4A5* gene of Xq22.3 in X-linked Alport syndrome, the *IDS* gene of Xq28 in mucopolysaccharidosis type II, the *RS1* gene of Xp22.2-p22.1 in X-linked juvenile retinoschisis, and the *DMD* gene of Xp21.2 in Duchenne muscular dystrophy.[49,80,81] Moreover, Bowl et al.[82] identified a large insertion on chromosome Xq27.1 in X-linked recessive hypoparathyroidism patients.

2. *CNV and common human diseases/complex traits*: Several common complex human diseases, including obesity, schizophrenia, autism, and cancer, have been shown to be associated with CNVs.[37]

3.1 Obesity

Obesity is a serious health problem with strong genetic determination, characterized by a high BMI. Several studies have been performed to analyze associations between CNVs and obesity. For example, a common deletion near the *NEGR1* gene was determined to be associated with BMI in a metaanalysis of 15 GWASs comprising more than 32,000 individuals.[83] This result was replicated by another study in nearly 300,000 subjects using the same approach.[98] Interestingly, a research focused on severe early-onset obesity identified another 43-kb deletion of *NEGR1*, partially overlapped with reported segment, significantly associated with severe obesity.[99] They also found that this signal was entirely driven by a flanking 8-kb deletion that provided a protective effect. *NEGR1* encodes neuronal growth regulator 1, which may be involved in cell adhesion. The protein encoded by this gene is a member of the type 3 G protein–coupled receptor family and may mediate the cellular effects of retinoic acid on the G protein signal transduction cascade.[100,101] However, there is no evidence for a direct connection between this gene and obesity. Neuropeptide Y receptor (pancreatic polypeptide 1, *PPYR1*) is a member of the seven transmembrane domain G protein–coupled receptor family. Peripheral administration of pancreatic polypeptide inhibits gastric emptying and decreases food intake in humans.[102,103] A CNV of *PPYR1* was detected in a Chinese population, thus suggesting that the CNV of *PPYR1* might be important for BMI variation.[84] However, a conflict is reported for deletion of *PPYR1* between human and mice.[104] For instance, null *PPYR1* mice have reduced body weight, but *PPYR1* gene deletion was associated with obesity.[84,85] This requires further studies for validation.

Using the Affymetrix Genome-Wide Human SNP Array 6.0, Jarick et al.[85] performed a GWAS between common CNVs and early-onset extremely obese children in 424 case–parent obesity trios, an independent sample of 453 extremely obese children and adolescents, and

435 controls. They found a novel CNV on chromosome 11q11 that was significantly associated with obesity. Wang et al.[105] performed another genome-wide CNV survey using 430 obese case subjects (BMI > 35) and 379 never-overweight control subjects (BMI < 25), and found that large CNVs are enriched in the obese individuals. The CNV that was found to be associated with PWS (chromosome 15q11–q13) has also been found to be associated with obesity in a study of 1000 unrelated US Caucasians.[86]

3.2 Schizophrenia

Schizophrenia is a highly heritable, psychiatric disease that often causes substantial social and occupational dysfunction.[106] Using high-density microarrays, two powerful large-scale association studies identified CNVs on chromosome 1q21.1 and 15q13.3 associated with schizophrenia.[87,88] These two CNVs were rare, with frequencies of ~0.1%–0.3% in schizophrenia cases and 10-fold less in control subjects. The sample sizes of these two studies were large and provided sufficient power to detect these rare CNVs. One study used 3,391 schizophrenic patients and 3,181 controls,[87] and the other [88] investigated 4,187 schizophrenic patients and 41,201 controls. A number of highly plausible schizophrenia candidate genes are located in these two regions. For example, at 1q21.1, the gene connexin-50 (*GJA8*), which encodes a gap junction subunit, was previously suggested as a schizophrenia gene.[107] Also, 15q13.3 contains another schizophrenia candidate gene, α7 nicotinic receptor gene *CHRNA7*.[108] Another large schizophrenia sample comprised of 6,882 cases and 11,255 controls was genotyped on Illumina arrays, aiming to discover novel susceptibility CNV loci.[109] However, after replication analysis in additional 14,568 cases and 15,274 controls, 12 distinct loci were identified with nominal levels of significance. There was another study performing cytogenomic array within 15,749 cases and 4,519 controls.[89] A rare deletion on chromosome 17q12 was identified to be significantly associated with schizophrenia. Moreover, 18 of 15,749 patients, but none of the controls, had this deletion. Fifteen genes are located in the deletion interval. Among them, *LHX1* is dosage sensitive and essential for normal brain development and function.

Besides the deleterious effect, large, rare CNVs could play a protective role in neurodevelopmental disorders as well. The largest CNV analysis of schizophrenia (47,005 individuals) to date reported that large duplications (1.5–3.0 Mb) at 22q11.2 were less common in schizophrenia cases than in the general populations (0.014% vs. 0.085%),[90] representing the first putative mutation for schizophrenia.

3.3 Autism

Autism spectrum disorders (ASDs) represent a neurodevelopmental disease characterized by impairment of social interactions, a restricted range of behaviors, and problems in communication.[110] In 2007, two landmark studies showed that de novo CNVs were important genetic factors for the pathogenesis of ASDs.[111,112] The Autism Genome Project Consortium used Affymetrix 10K SNP arrays to investigate 1181 families with at least 2 affected individuals, and found 154 CNVs in the autism cases.[112] By examining 264 families with at least one case of ASD, Sebat et al. [111] found that 10% of sporadic cases had de novo CNVs, compared with 3% of healthy members within case families, whereas 1% in control families had de novo CNVs. Marshall et al.[113] obtained similar results by analyzing 427 unrelated ASD cases via SNP microarrays and karyotyping. Weiss et al.[114] and Kumar et al.[91] determined that ~1% of patients with autism had common recurrent 600-kb deletions and duplications on 16p11.2. Using SNP microarrays and examining 2195 patients with ASD and 2519 healthy controls, Glessner et al.[92] determined that several new susceptibility genes, such as *NLGN1* and *ASTN2*, were enriched within CNV regions in cases compared to controls. Additionally, to estimate the effect of CNVs in the 1–30 kb range in an autism case–control sample, Poultney et al.[115] made use of the eXome Hidden Markov Model (XHMM) program to identify exonic CNV from whole-exome sequencing data, and observed significant increase in the burden of rare CNVs in ASD. eXome Hidden Markov Model is an approach that uses principal component analysis to normalize exome read depth and a Hidden Markov Model to identify exonic CNVs.[116]

3.4 Cancer

Although the exact pathogenic mechanisms leading to many cancers are unclear, the consensus view is that cancer results from dysregulation of the activity or expression of genes that control cell growth and differentiation, leading to abnormal cell proliferation.[117] CNVs have been reported in many kinds of cancers,[18] and presumably contribute to this dysregulation.

Using high-resolution SNP arrays, Mullighan et al.[93] performed a genome-wide analysis of leukemic cells from 242 pediatric patients with acute lymphoblastic leukemia (ALL). They found copy number changes in *PAX5*, a gene within the B-cell development pathway, in 57 of 192 ALL cases. CGH analysis has also shown that CNVs are associated with prostate cancer,[118] breast cancer, and colorectal cancer.[119] Utilizing genome-wide SNP-based arrays, Tse et al.[94] identified eight regions with CNVs including six deletions (on chromosomes 3, 6, 7, 8, and 19), and two duplications (on chromosomes 7

and 12) that were significantly overrepresented in nasopharyngeal carcinoma (NPC) patients compared with healthy controls. Among these CNVs, *MICA* and *HCP5* gene deletions on chromosome 6p21.3 showed the highest association signal. Examining 51 BRCA1-associated ovarian cancer patients, and 47 healthy women via Affymetrix Genome-Wide Human SNP Array 6.0, Yoshihara et al.[120] identified germline CNVs in BRCA1-associated ovarian cancer patients. Besides, in the year of 2012, as part of The Cancer Genome Atlas (TCGA) project, a comprehensive study of lung squamous cell carcinoma was conducted, and a landscape of genomic and epigenomic characteristics, including CNVs identification, was descripted subsequently.[95] Using the SNP 6.0 array platform and GISTIC 2.0, they identified 50 peaks of significant amplification or deletion regions, among which included the regions reported for the first time, including amplifications of *NFE2L2*, *MYC*, *CDK6*, *MDM2*, *BCL2L1*, and *EYS* and deletions of *FOXP1*, *PTEN*, and *NF1*.

3.5 Congenital Heart Disease

Congenital heart disease (CHD) usually refers to abnormalities in the heart's structure or function that arise before birth,[121] and affects 0.8% of live birth.[122] The presence of mutations in regulators of heart development during embryogenesis is reported as the major cause of CHD,[123] which relies on the identification of *TBX5*, *NKX2-5*, *GATA4*, *TBX1*, and *SALL4* one after another.[121]

Several studies have shown that rare de novo and inherited copy number variants were enriched in classified CHD patients. For example, Warburton et al. used the NimbleGen HD2-2.1 CGH platform to identify CNVs from 223 consecutively ascertained families.[96] They established 12 CNVs and several implicated genes that were likely causes of CHD after fluorescent in situ hybridization and qPCR confirmation, including *NODAL*, *CFC1*, and *NOMO3*, which have interaction in the nodal pathway. Similarly, Erdogan et al.[124] performed a submegabase resolution array CGH on 105 patients with CHD, as well as subsequent qPCR and fluorescent in situ hybridization verification. As the result, three de novo and eight inherited CNVs were identified, which provide the elucidation of the pathogenetic mechanisms underlying CHD. Additionally, Sailani et al.[125] reported genomic variations that determine the risk for CHD in DS. It is said that CHD occurs in 40% of DS cases.[125] The authors performed a case–control GWAS study, including 187 DS with CHD as cases, and 151 DS without CHD as controls. Finally, two regions were successfully replicated, which are both located at chromosome 21. The result highlights the potential role of the CNV architecture on the CHD risk of DS.

4 CNV AND OSTEOPOROSIS

CNVs have also been found to be associated with susceptibility to Parkinson disease, Alzheimer disease, HIV infection, and Crohn disease.[18,37] However, few studies have been performed to detect the association between CNVs and bone-related diseases.[11,126,127] In this section, we discuss in detail the relationship between CNVs and the most common bone diseases, osteoporosis (OP).

OP is the most common metabolic bone disease in humans, generally characterized by low–bone mineral density (BMD), poor bone quality (characterized by bone geometry etc.), and/or increased susceptibility to low-impact osteoporotic fractures (OF). It is widely accepted that variation in BMD and bone geometry, and risk for OFs, are largely determined by genetic factors, but the overwhelming majority of genetic variation accounting for OP risk has not been determined.[128] Previous investigations,[128] including recent GWASs,[129–135] have focused on SNPs, and have shown that a number of genes or SNPs appear to enhance the risk of developing OP. Collectively, however, all of these implicated genes or SNPs account for only a small portion of the risk of OP. Consequently, it has become important to explore whether some remaining undiscovered genetic factors that influence risk of OP are due to genomic mechanisms other than individual mutation changes, such as CNVs.

The first investigation of CNVs in OP was performed by Yang et al.[11] who studied 350 elderly Chinese Han subjects with a history of hip OF and 350 healthy age-matched controls. They constructed a genomic map containing 727 CNV regions, using Affymetrix Human Mapping 500K Arrays. Subsequently, 116 common CNVs with frequencies exceeding 1% were detected. CNV 4q13.2 was determined to have a significant distribution difference between patients and controls, with a P value of 2.0×10^{-4}. Five protein-coding genes, *UGT2B17*, *YTHDC1*, *TMPRSS11E*, *TMPRSS11E2*, and *UGT2B15*, are located within CNV 4q13.2 and its flanking region. After fine mapping, these investigations found that gene copy number deletions were only observed for *UGT2B17*. Subjects with one or two copies of *UGT2B17* had a greater risk of OF than those with homozygous deletions. These results were replicated in another Chinese sample of 399 patients with hip OF and 400 controls. They further examined this CNVs relevance to major risk factors for OF (i.e., hip BMD and femoral neck bone geometry, i.e., cortical thickness and buckling ratio) in both Chinese and Caucasian DNA samples, and found that copy number deletions of *UGT2B17* are much more common in Chinese than in Caucasians. Further, although the signal of association tests was less significant in

Caucasians than in Chinese, the relationship between CNV of *UGT2B17* and risk of OP exhibited the same trend within both Chinese and Caucasian populations, that is, higher *UGT2B17* gene copy numbers were associated with lower BMD, thinner cortical thickness, higher buckling ratio, and an increased risk of OF in both populations. The *UGT2B17* gene encodes a key enzyme responsible for glucuronidation of androgens and their metabolites in humans.[136,137] Androgen is a major source for estrogen, and both androgen and estrogen have direct effects in stimulating bone formation.[138] The authors also examined the relationship between serum total testosterone or estradiol levels and the *UGT2B17* gene copy number in 236 Chinese young men. Testosterone and estradiol concentrations were significantly reduced in the men with homozygous deletions of *UGT2B17*.

Subsequently, Chew et al.[126] genotyped *UGT2B17* copy number in 1347 elderly Caucasian women, and in contrast found no evidence of an effect of *UGT2B17* CNV and OP risk. These conflicting results might be attributed to differences in patient populations and the differences in CNVs between these populations. For example, in the Caucasian sample of 1000 unrelated subjects, Yang et al.[11] determined that 11.9% of the study subjects had a homozygous deletion of *UGT2B17*, 44.4% had a heterozygous deletion, and 43.7% had two copies. In marked contrast, in the Chinese DNA samples, 76.8% had homozygous deletions of *UGT2B17*, 21.6% had heterozygous deletions, and 1.6% had two copies.[11] In the study of Yang et al., the association signal between *UGT2B17* CNVs and risk factors for OP was only moderate in Caucasians.[11] Consequently, it is not totally unexpected that Chew et al.[126] did not detect this association in Caucasians.

Deng et al.,[127] using the same 1000 unrelated Caucasian subjects studied by Yang et al.,[11] detected a CNV in the *VPS13B* gene that was significantly associated with spine, hip, and femoral neck BMD, as well as bone geometry at the femoral neck.

Later in 2013, Oei et al. performed another genome-wide CNV association study in 5178 individuals from a prospective cohort in The Netherlands and executed in silico lookups, as well as de novo genotyping to replicate.[139] As the result, they successfully observed a rare 210-kb deletion located on chromosome 6p25.1 significantly associated with the risk of fracture. However, this signal was indicated to be predisposed to higher risk of fracture only in the population of European origin.

Collectively, these studies lend fairly strong support for the conclusion that CNVs can contribute to the genetic predisposition to develop OP. Further studies are needed to validate the relationship between CNVs and OP.

5 CONCLUSIONS

CNVs represent regional chromosomal mutations. Therefore, they contain more sequence information and have higher per-locus mutation rates than SNPs. Likely, they are responsible for changes in gene function and genome evolution via gene deletion, duplication, or exon shuffling. Studies of CNVs in human disease have progressed rapidly. CNVs play an important role in a range of human disorders, but these CNVs can be rare, common, or de novo. However, the mechanism(s) by which CNVs alter susceptibility to diseases remains unknown, but will undoubtedly be the subject of future investigations.

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CHAPTER

5

Genomic Profiling in Bone

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1 INTRODUCTION

This year marks the 14th anniversary of the completion of the Human Genome Project,[1,2] a landmark achievement that has provided the road map for human genetics to query every base pair against possible functional attributes contributing to tissue patterning, metabolism, or disease. While the advantages of embracing genomic technologies to advance our understanding of gene function and association to disease may seem obvious, many scientific disciplines have lagged behind in adopting genomic approaches to further their scientific studies. A PubMed query using the keyword "genomics" returns ~150,000 entries; "bone genomics" contributes less than 1% in contrast to "cancer genomics" which is the most populous category representing >17% of this total collection. Of ~2100 publications associated with the keyword "bone genomics" >50% (~1200) of them were published in the past 5 years, suggesting that the bone biology field is making rapid progress in embracing the new genomic technologies. However, more specialized searches, such as "osteoblast genomics" or "chondrocyte genomics," narrow the search to 235 and 170 publications, respectively, highlighting the need for the field of skeletal biology to more aggressively adopt genomic methods and technologies.

Generating the sequence of human and other model organisms' genomes has helped define the precise location of all known genes. Bioinformatic tools have helped predict novel genes, alternative isoforms, and protein domains, but have provided little guidance concerning the putative function of novel transcripts. In addition to defining protein domains that may be associated with known cellular functions, quantifying transcript expression across diverse cell types is critical for understanding human biology and this task is far from being exhausted. Until recently, production of such data was prohibitively expensive and experimentally laborious. The major method for annotating a transcriptome required the slow and costly process of cloning cDNAs or expressed sequence tag (EST) libraries, followed by capillary sequencing.[3–5] Owing to the high costs and limited data yield intrinsic to this approach, it only provided a glimpse of the true complexity of cell type–specific splicing and transcription. Analysis of these data required sophisticated computational tools, many of which provide the basis for the programs used today for high-throughput RNA sequencing (RNAseq) data.[6] Alternative strategies, such as genome-wide tiling arrays, allowed for the identification of transcribed regions at a larger and more cost-efficient scale but with limited resolution. Splicing arrays with probes across exon–exon junctions enabled researchers to analyze predefined splicing events but could not be used to identify previously uncharacterized events.[7]

The entire repertoire of transcripts in a cell, tissue, or organism, also known as the *transcriptome,* represents the connecting bridge of information that links what is encoded in our DNA to the phenotypes that are driven by genetics, environment, and disease. Therefore, a better understanding of the transcriptome will facilitate the extrapolation of genotypes to phenotypes and enhance our ability to discover biomarkers that can accurately predict emerging phenotypes as a function of disease progression. The tools for examining RNA levels have been available for many decades through the use of northern blots, reverse-transcription PCR, ESTs, and serial analysis of gene expression.[8] It was, however, the development of gene expression microarrays in the late 1990s that expedited our ability to survey many gene transcripts in parallel.[9–11] More recently, direct sequencing of transcriptomes has allowed for a fully unbiased and unrestricted characterization of all transcribed sequences within a given sample.[6]

In the field of skeletal biology, most "genome-scale" progress has occurred in the area of genome profiling or expression quantification,[12] using gene-expression

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microarrays. Microarrays come in two flavors: cDNA or oligonucleotide arrays. The main distinction between the platforms is that cDNA microarray are home manufactured by printing full-length cDNA spots in an investigator's facility,[13] while the other platforms are commercially mass produced (Affymetrix, NimbleGen, Qiagen, Agilent, Illumina) through printing of high-density short oligonucleotides.[14] For example, the newest Affymetrix GeneChip Human Gene ST Array provides coverage for >30,000 coding transcripts and >11,000 long intergenic noncoding transcripts. While this process is limited to studying the expression of validated transcripts for defined isoforms, it does however provide a glimpse of the repertoire of "genes" that are transcribed as a function of cell type, or biological process, and genome profiling in this field has led to some intriguing discoveries. Recent advances in RNAseq technology have enabled us to overcome several limitations of microarrays; it provides a powerful tool to decipher gene expression changes at a global scale, allowing the detection of novel transcripts, allele-specific expression, single nucleotide variants, and indels. However, skeletal biology is far behind in embracing the potentials of this technology compared to other fields, such as cancer biology.

Comprehensive analysis of gene expression patterns and regulatory networks involved in skeletal development and remodeling is a prerequisite to completely understand physiological bone structure, function, and homeostasis. It also has a crucial role in the development of appropriate therapeutic strategies for various diseases affecting the skeleton. Here we will review the genome profiling body of work that has been generated in the last decade, with emphasis on: (1) skeletal cell type gene expression; (2) spatial and temporal differences in skeletal gene expression; (3) profiling biomechanical effects on bone; (4) gene expression in osteoporosis (OP) and other bone mineral density (BMD) animal models; (5) gene expression profiling in patients with OP, and (6) profiling noncoding RNA expression in bone. While many profiling reports have focused on in vitro models of cell differentiation, mineralization, or signaling perturbations, here we will primarily focus on literature that describes profiling of homogenous cell samples, tissue microdissection or organ isolation from live animals, or human samples to provide a snapshot of genomic profiling that may more faithfully adhere to in vivo bone physiology. The data described later is also summarized in Table 5.1.

1.1 Profiling Skeletal Cells and Bone Metabolism

Three dominant cell types maintain the function of the adult mineralized bone: osteoblasts, osteocytes, and osteoclasts (Fig. 5.1). Osteoblasts are responsible for the synthesis, deposition, and mineralization of extracellular matrix. Osteocytes are osteoblasts that have become embedded within the extracellular matrix, residing in cavities termed lacunae.[41] These cells communicate with each other, as well as with osteoblasts on the bone surfaces by means of cytoplasmic extensions that extend through long, intricate channels called canaliculi that are present throughout the mineralized bone. Osteoclasts are large multinucleated cells that reside on bone surfaces and function primarily to resorb bone through direct chemical and enzymatic reactions. A minority of cells in the bone are represented by undifferentiated mesenchymal stem cells (MSCs) that reside in the bone marrow, along vascular channels, and in the condensed fibrous tissue covering the outside of the bone, the periosteum. MSCs give rise to the osteoblast and osteocyte progenitors while osteoclasts are derived from hematopoietic stem cells housed in the bone marrow, or spleen.[42]

The principal role of osteoblasts is to form new bone via the synthesis of various proteins and polysaccharides that assist or contribute to the formation of mineralized bone matrix. Other functions include the regulation of bone remodeling and mineral metabolism. Studies of osteoblastic cells isolated from trabecular bone, embryonic calvaria, and osteosarcoma have established a set of molecular makers that help scientists distinguish the osteoblast phenotype. These markers involve the synthesis of type I collagen, the expression of alkaline phosphatase (ALP), the secretion of osteocalcin, and the production of mineralized matrix. While individually these markers are not unique to osteoblasts, as a cohort they are universally accepted in evaluating the osteoblast's identity or function. Osteoblasts also express receptors for various hormones including parathyroid hormone (PTH), 1α,25-dihydroxyvitamin D_3 [1α,25(OH)2D_3], estrogen, and glucocorticoids, all of which are also involved in regulating osteoblast differentiation. Over the years, much attention has been given to commitment of osteoprogenitor cells, lineage progression, and differentiation into osteoblasts, processes that have been studied in various cell lines[43–46] and primary cultures derived from humans and rodents.[47–50] These studies reported a generalized pattern of suppression of genes associated with differentiation of mesenchymal progenitors into other cell lineages, including myocytes and adipocytes, induction of genes associated with cell proliferation followed by matrix maturation, and mineralization. However, the gene expression profiles obtained from in vitro osteogenic differentiation experiments represent only an approximation of changes occurring in vivo, which necessitates their validation in appropriate in vivo models.

Osteocytes represent terminally-differentiated osteoblasts. They are the most abundant cellular component of mature mammalian mineralized bones and constitute

TABLE 5.1 Summary of Differentially Expressed Genes Identified in the Studies Described Here

Functions	Models	Cells/tissues	Platform	No. of differentially regulated genes	Genes[a]	References
Bone remodeling	Transgenic mice: Col2.3GFPcyan, Dmp1GFPtopaz	Osteoblasts/ osteocytes	Illumina MouseWg-6v1 BeadChip	Osteocyte versus osteoblast: 249 upregulated, 136 down regulated	**Up**: *Col15a1, Col18a1, Col4a1, Col22a1, Cola2, Col16a1, Col27a1, Col3a1, Col9a2, Col9a1, Col8a2, Col12a1, Col14a1, Col2a1, Col8a1, Dmp1, Bmp4, Bmp8a, Bmp3, Dkk1, Notch1, Notch3, Dlk1, Pitx2, Tbx3, Irx5, Irx6* **Down**: *Mmp9, Mmp23, Adamts18, Reln, Tingal, Htra1, Prss12, Cpxm1, Cfb, Kera, Acan, Prg4, Hapln1, Aspn, Chad, Fmod, Fbln1, Fbln2, Thsb2, Thsb3, Spon1, Comp, Prelp, Mant1, Postn, Wnt9a, Wnt10a, Dkk3, Gdf10, Ptn, Zic1, Gsc, Runx1t1, Pax1, Scx*	Paic et al.[15]
Anatomical location dependent differences in gene expression	Rat ulnar limb and parietal skull bone	Total bone RNA and osteoblasts	Affymetrix GeneChip	Skull bone versus limb: 1236 differentially expressed genes. Skull bone-derived osteoblasts versus limb derived osteoblasts: 249 genes	**(Skull vs Limb) Up**: *Opg, Pthr1, Calcr, Lrp5, Sost, Ibsp, Bmp3, Bmp6, Cthrc1, Ptn, Wif1, Sfrp2, Vdr, Prelp, Gpnmb, Tcf7, Nog, Cthrc1, Csf1, Mmp9, Fos, Dlx5, Dlx3, Tbx2 and Ctsk*. **Down**: *Comp, Mmp8, Ctnnb1, Wnt16, Bmp5 and Hoxa5, Hoxa7, Hoxa10, Hoxc5, Sox6, Gata1, Gata3, Cited4*. **(Skull bone-derived osteoblasts vs limb derived osteoblasts) Up**: *Sfrp2, Apoe, Irx5, and Msx2*. **Down**: *Hoxa1, Hoxa4, Hoxa5, Hoxa7, Hoxa10, and Hoxa13*	Rawlinson et al.[16]
Anatomical location dependent differences in gene expression	Rat mandible, ulna, and calvaria	Total bone RNA	Affymetrix GeneChip	Common to mandible and calvaria, but not ulna: 1014. Common to mandible and ulna, but not calvaria: 873	**Common to mandible and calvaria, but not ulna**: *Fos, Maf, Myo6, Smad3, Gata6, Ankrd1, Gli1, Nfatc4, Sox2, Gli2, Tcf7, Bmpr2, Irx2, Irx4, Klf15, Klf9, Dlx3*. **Common to mandible and ulna, but not calvaria**: *Pax6, Tnf, Cdh1, Cited4, Shox2, Myog*	Kingsmill et al.[17]
Age dependent differences in gene expression	Needle bone biopsies from the posterior iliac crest of young and old women	Total bone RNA	RNAseq	Old versus young subjects: 446 up; 232 down	**Up**: *S0X17, S0X7, FZD4, CDH3, SFRP1, S0X18, S0X13, CCND1, N0TCH3, N0TCH4, JAG2, DLL4, HES1, HEY1*. **Down**: *S0X4, SFRP5, LEF1*	Farr et al.[18]
Mechanical loading	Mouse tibia subjected to ankle loading	Total bone RNA	Agilent Technologies	22 > 2-fold up 15 < 2-fold down	**Up**: *Mmp3, Has, Mrgpra2a, Thbs3, Timp1, Cola1, Matn2, Matn4, Cdz48, Cspg4, Fosb, Aspn, Cdh13, Aqp1, Moxd1, Anxa8, Atf3, Aebp1, Prrx2, Cma1, Hapln1, Il1*. **Down**: *Btc, Senp6, Crrc34, Barhl2, Tnfp, Phf20l1, Kirrel3, Crisp1, Npy2r, Krt82, Ptgs2, Pax7a, Tnf236, Crlf1*	Zhang et al.[19]

(*Continued*)

TABLE 5.1 Summary of Differentially Expressed Genes Identified in the Studies Described Here (*cont.*)

Functions	Models	Cells/tissues	Platform	No. of differentially regulated genes	Genes[a]	References
Mechanical loading	Rat ulna subjected to axial loading	Total bone RNA	Affymetrix GeneChip	Differentially regulated genes (4 hours, 12 hours, 1 day, 2 days, 4 days, 6 days, 8 days, 12 days, 16 days, 24 days, and/or 32 days) 1051	**Early response genes**: *Fosl1, Junb, Ccl2, Ccl7, Cxcl1, Cxcl13, Osm, Osmr, Adamts1, Serpina3n, Serpine1, Lep, Sdc4.* **Matrix formation (Up):** *Alpl, Bglap, Col1a2, Cthrc1, Fn1, Ibsp, Lox, Sparc, Vcan, Bgn, Fgf14, Pdgfa, Pdgfc, Pdgfrl, Pgf, Wisp1, Pthr1, Sp7*. **Matrix formation (Down)**: *Efemp1, Mmp8, Prelp, Serpinb2, Spon1, Egf, Fgf1, Fgf7, Fgf23, Fgl2, Hgf, Sost, Sfrp4, Chrdl1, Grem1*	Mantila Roosa et al.[20]
Mechanical loading	Mouse vertebra subjected to controlled compression loading	Osteocytes	Affymetrix GeneChip	Single dose loading: 287 up, 52 down Repetitive loading: 778 up, 561 down	**(Single dose loading) Up**: *Wnt5a, Aspn, Igf1, Emilin2, Ccl12, Adamts4, Adamts1, Serpina3c, Stc1.* ***Down***: *Olfm3, Spock2, Serpin9b9.* **(Repetitive loading) Up**: *Ptn, Vcan, Aspn, Thbs4, Sema3c, Sema3d, Thbs2, Thbs1, Mmp2, Cyr61, Grem1, Fgf18, Grem2, Pdgfd, Wnt5a, Dmp1.* **Down**: *Ltbp1, Muc4, Apoa5, Adamts19*	Wasserman et al.[21]
Mechanical loading	Mouse tibia subjected to mechanical loading	Cortical and cancellous bone RNA	RNAseq	3 h After loading: 43 genes in cortical bone and 18 in cancellous bone 24 h after loading: 58 genes in cortical and 32 genes in cancellous bone	**(3 h) Up**: *Wnt1, Wnt7b, Timp1, Ptgs2, Opg*. **Down**: *Sost, Dkk1, Lrp5* **(24 h) Up**: *Ptn, Vcan, Cthrc1, Hapln4, Tubb3, Wnt1, Wnt10b*. **Down**: *Myh4, Tnnc2, Smpx, Tnnt3, Mylk2, Myoz1, Myom2, Actn3, Myl1, Casq1, Mybpc2, Myh2, Mylpf, Tnni2*	Kelly et al.[22]
Animal model of OP	OVX-induced OP, non-OP, aged, and juvenile rats	Bone marrow mesenchymal stromal cells	Agilent Technologies	OP versus normal: 195 up, 109 downregulated. Aged versus normal: 62 up, 86 downregulated. Juvenile versus normal: 120 up, 80 downregulated	**(OP vs. Ctrl) Up**: *Spon1, Alox5, Baat, Sult4a1, Lp1, Mmp8, Cyp3a9, A2m, Alp1, Crabp2, Slc26a1, Cdkn2b*. **Down**: *Npy, Cd14, Cd24, Ramp3, Marcks11, Wnt4, Plpcb, Adrb3*. **(OP vs. Aged) Up**: *Mmp8, Spon1, Csrp2, Ivl, Folr1, Crabp2, LOC64305, Nac1, Gludins, Braf, Inhbp, Pgr, Slc26a1, Sp1*. **Down**: *Prlpb, Iilrn, Plpcb, LOC171569, Ramp3, Mip*	Xiao et al.[23]
Animal model of OP	Runx KO mouse embryonic skeletal tissues from calvaria, forelimbs, and hindlimbs versus WT control	Mouse skeletal tissues	Affymetrix GeneChip	Calvaria: 531 down, 746 up. Forelimb: 296 down, 310 up. Hindlimb: 172 down, 320 up. All three tissues: 25 down, 41 up	**Up**:*Reln, Penk1, Dlk1, Figf, Mmp8, Trim2*. **Down**: *Ibsp, Spp1, Pim1, Satb2, Akp2, Mmp9, Snf1lk, Col9a1, Runx2, Agc1, Matn4, Scgf, Dlx5, Cfh, Gtpbp2, Tubb5, Hck, Tce1, Ppp2r5d, Ndufb10, Fdps*	Vaes et al.[24]

Animal model of OP	Integration of human genomic expression and inbred mouse gene expression profiles	Total bone RNA	Agilent Technologies	148 candidate genes correlated with BMD, 18 of which were casually linked to differences in BMD	**Correlated genes**: *Grem2*, *Twist2*, *Ccdc28b*, *Timd4*, *Smad4*, *Rasd1*, *Wnt9a*	Hsu et al.[25]
Animal model of OP	Integration of human genomic expression and inbred mouse gene expression profiles	Total bone RNA	Affymetrix GeneChip	16 candidate genes linked to differences in BMD	**Correlated genes**: *Gpr177*, *Hecw2*, *Casr*, *Mmrn1*, *Irx2*, *Pdzd2*, *Tgfb1*, *Cacnb2*, *Dock1*, *Sox6*, *Pdgfd*, *Rad51l1*, *Sall1*, *Fbxo31*, *Cdh2*	Farber et al.[26]
Animal model of OP	Tibia from Lrp5 mutant mice	Total bone RNA	RNAseq	$Lrp5^{-/-}$ versus $Lrp5^{+/+}$: 302 genes $Lrp5^{-/-}$ and Lrp5p.A214V/+: 166 genes Lrp5p.A214V/+ and $Lrp5^{+/+}$: 28 genes	**($Lrp5^{-/-}$) Up**: *Wnt10b*, *Fzd4*, *Postn*, *Thbs2*. **Down:** *Col1a1*, *Col1a2*, *Bglap*, *Mt1*, *Mt2*, *Cyr61*, *Cgref1*, *Gata1*. **($Lrp5^{p.A214V/+}$ *vs.* $Lrp5^{+/+}$) Up**: *Kera*, *Myoc*, *Angptl7*, *Serpina1a*, *Serpina1d*, *Serpina1b*, *Serpina1e*. **Down**: *Igkv9-123*, *Igkv1-88*, *Igkv8-27*, *Ighv7-1*	Ayturk et al.[27]
Animal model of OP	Vertebra from rats treated with Sost antibody (single dose, 100 mg/kg)	Osteoblasts, LC, and osteocytes (6, 24, 72, and 168 h posttreatment)	Affymetrix GeneChip	The number of probe sets changed in response to Sost antibody in any cell type or time point ranged from 0 to 514	**Genes differentially regulated in response to acute Sost antibody treatment**:*Wisp1*, *Twist1*, *Bglap*, *GJa1*, *Tcf7*, *Enpp2*, *Mmp2*, *Omd*, *Bgn*, *Dcn*, *Sparc*, *Lepre*, *Lox*, *Bmp1*, *Tmem119*, *Pdgfa*, *Cxcl12*, *Cxcl14*, *Dlx5*, *Smpd*, *Sgms2*, *S1pr3*, *Cdkn1a*, *Cgref1*, *Aurka*, *Bub1*, *Ccnd1*	Nioi et al.[28]
Animal model of OP	Vertebra from rats treated with Sost antibody (3 or 50 mg/kg/week for up to 26 weeks)	Osteoblasts, LC, and Osteocytes (8, 29, 85, 183, 237, and 309 days posttreatment)	Affymetrix GeneChip	In osteoblasts, 1650 genes clustered into 7 modules. In lining calls, 291 genes clustered into 3 modules. In Osteocytes 1839 genes clustered into 6 modules	**Genes differentially regulated in response to chronic Sost antibody treatment**: *Wisp1*, *Twist1*, *Cd44*, *Id2*, *Irx3*, *Vegfa*, *Tcf7l2*, *Wnt5b*, *Sfrp4*, *Birc5*, *Ccnd1*, *Mmp9*, *Cdkn1a*, *Mdm2*, *Aurka*, *Bub1b*, *Chek1*, *Kif23*, *Kifc1*, *Knstrn*, *Plk1*, *Dmp1*, *Mepe*, *Phex*, *Postn*, *Tgfb3*, *Bmp7*, *Col1a1*, *Alpl*, *Mgp*	Taylor et al.[29]
Human OP/ BMD	Isolated circulating monocytes from premenopausal women with high and low PBM	Monocytes	Affymetrix GeneChip	Low versus high PBM: 37 upregulated and 12 down regulated	**Up**: *Serping1*, *Sco2*, *LOC440607*, *Blvra*, *Sat*, *Ubc2l6*, *Tnfsf13b*, *Irf7*, *Gbp2*, *Epsti1*, *1l15*, *Scarb2*, *Mafb*, *Rsn*, *Parp14*, *Sp110*, *Gch1*, *Tnfsf10*, *Lyn*, *Mx2*, *Pstpip2*, *114l1*, *Lap3*, *Plscr1*, *Napsb*, *Ncoa7*, *Ifi44*, *Hsxiapaf1*, *Parp12*, *Glul*, *Wars*, *Marcks*, *Map17*, *Stat1*, *Gbp1*, *Cxcl10*, *Fh*, *Lrmp*. **Down**: *Supt5h*, *Ecgf1*, *Ifit2*, *Fth1*, *Sash1*, *Mrpl30*, *Mthfr*, *G1p3*, *Napa*, *Asah1*, *Ecm1*	Lei et al.[30]

(*Continued*)

TABLE 5.1 Summary of Differentially Expressed Genes Identified in the Studies Described Here (*cont.*)

Functions	Models	Cells/tissues	Platform	No. of differentially regulated genes	Genes[a]	References
Human OP/BMD	Isolated circulating monocytes in postmenopausal women with high and low BMD	Monocytes	Affymetrix GeneChip	Low versus high hip BMD	**Key genes**: *DAXX*, *PLK3*, *PDCD5*, *VDAC1*, *HBP1*, *CDKN2D*, *GAS2L1*, *PKD1*, *PPP1R15A*	Liu et al.[31]
Human OP/BMD	Isolated human osteoblasts from osteoarthritis and osteoporotic patients	Osteoblasts	Agilent Technologies	OP versus non-OP: 144 up and 208 down regulated genes	**Up**: *Col1a1*, *Mgp*, *Tnfrsf11b*, *Cxcl2*, *Adra2a*, *Bmp3*, *Tspan8*, *Slc7al11*, *Cnnd2*, *Psg11*, *Col15a1*. **Down**: *Bmp2*, *Ramp3*, *Runx2*, *Alpl*, *Csf1*, *Ptn*, *Ibsp*, *Prpf19*, *Col10a1*, *Ngef*, *Ccnb2*, *Cdc2*, *Pbk*. **Other important differentially expressed genes**: *Rpl13a*, *Cdkn1a*, *Cel2*, *Cxcl2*, *Csfl1*, *Tnfrsf19*, *Arpc1a*, *Arpc1b*, *Ppp1r12a*, *Actn1*, *Thoa*, *Arpc5*, *Sfrp2*, *Ccnd2*, *Csnk2a2*, *Ppp2ca*, *Ctbp2*, *Tcf7*, *Prickl1*, *Rhoa*, *Fzd5*	Trost et al.[32]
Human OP/BMD	Human osteoarthritis versus OP versus normal bone	Human trabecular bone from intertrochanteric region of proximal femur	Adelaide Microarray facility	OP versus normal: 150 differentially expressed genes	**Up**: *Trem2, Anxa2, Scarb2, Ccl2, Ccl3, Cd14, St14, Ccr1, Adam9, Ptk9, Sgk1, Ctsb, Fst, Lgals1, Spp1, Sparc, Il10, Marco, Cd14, Ppp2cb, Mgl1, Rank, Shox2, Pdlim4, Hdac4, Taz*. **Down**: *Mnda, Pstp1p1, Adora2a, Lbr, Dok4, Nipbl, Nrg2, Bre, Mal, Ship1, Adora24, Irak2*	Hopwood et al.[33]
Human OP/BMD	Postmenopausal women with BMD variations: OP versus osteopenia versus normal	*Trans*-iliacal bone biopsies	Affymetrix GeneChip	142 Differentially expressed genes with strong correlation to BMD differences	**Up**: *Dkk1*, *Sost*, *C10rf61*, *Sec61b*, *Wil1*, *Pcgf1*, *Mepe*, *Slc13a4*. **Down**: *Acsl3*, *Nips-Nap3b*, *Abca8*, *Dleu2*, *Ktn1*, *Cops4*, *Lrp12*, *Ppp3cb*	Reppe et al.[34]
Human OP/BMD	Postmenopausal women with distinct BMD differences: osteoporisis versus intermediate versus normal	Hip, lumbar spine, and femoral neck bone biopsies	Affymetrix GeneChip	OP versus normal: 439 upregulated genes and 170 downregulated genes	***(All 3 tissues)*** **Up**: *Sost*, *Dkk1*, *Pggf1*, *Ppme1*. **Down**: *Ktn1*, *Ptp4a1*, *Rtn4*, *Slc16a1*, *Cops4*. ***(Femoral neck & spine)*** **Up**: *Bcl2*, *Sox4*.	Jemtland et al.[35]

Profiling miRNA expression in bone	Osteoporotic versus nonosteoporotic patients	Serum and bone	miRNA PCR Array	OP versus normal (serum): 11 OP versus normal (bone): 6	**Up**: *miR-21-5p*, *miR-23-3p*, *miR-24-3p*, *miR-25-3p*, *miR-27a-3p*, *miR-100-5p*, *miR-122a-5p*, *miR-124-3p*, *miR-125b-5p*, *miR-148a-3p*, *miR-223-3p*	An et al.[36]
Profiling miRNA expression in bone	OVX versus sham-operated mice	Femurs and tibias	Agilent Technologies	OVX versus sham-operated mice: 9 miRNA	**Up**: *miR-127*, *−133a*, −133a* −, *−133b*, *−136*, *−206*, *−378*, *and* −378*. **Down**: *miR-204*	Waki et al.[37]
Profiling miRNA expression in bone	Healing femoral shaft fractures compared versus unhealing fractures	Femurs	miRCURY LNA Array (Exiqon)	Healing fractures versus unhealing fractures: 317 up	**UP**: *miR-140-3p*, *miR-140-5p*, *miR-181a-5p*, *miR-181d-5p*, *miR-208b-3p*, *miR-451a*, *miR-743b-5p*, *and miR-879-3p*	Seeliger et al.[38]
Profiling IncRNA expression in bone	Osteogenic differentiation of human MSC	MSCs (day 0 and day 14)	IncRNA + mRNA human gene expression microarray V4.0 (CapitalBio Corp, Beijing, China)	Day 14 versus day 0: 687 up, 519 down	**Up**: *H1 9*, *uc022axw.1*, *ENST00000436742.1*. **Down**: *LINC00707*, *XLOC_008374*	Wang et al.[39]
Profiling IncRNA expression in bone	BMP2-induced osteoblast differentiation of C3H10T1/2 cells	BMP2 treated C3H10T1/2 cells and untreated cells (l day, 4 day)	Mouse IncRNA Array V2.0 (Arraystar)	BMP2 treated versus untreated (day 1): 886 up, 825 down (day 4): 595 up, 548 down	***(Day 1 and day 4)*** **Up**: *uc009odu.1*, *uc009gzn.1*, *uc009bxq.1*, *uc008drt.1*, *AK144695*, *BC023483*, *uc007djg.1*, *uc007cyp.1*, *NR_027652*, *CB272499*, *AV570737*, *AK201409*, *AK043290*, *AK032849*, *AK032137*, *AK030568*, *mKIAA0031 NR_033578*. **Down**: *uc009rnr.1*, *uc009qfj.1*, *BC099572*, *AK044623*, *mD53*, *uc007cpz.1*, *BC038927*, *AK142678*, *C130026I21Rik*, *BC038927*, *AK137923*	Zuo et al.[40]

BMD, Bone mineral density; LC, lining cells; OP, osteoporosis.
a Not all data have been fully respresented from each reference; for a full list of genes altered see referenced literature.

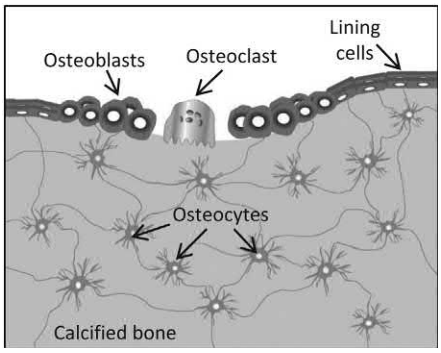

FIGURE 5.1 Cell types of the bone. Osteoblasts and osteoclasts reside on bone surfaces, where the osteoblasts secrete extracellular matrix and contribute to osteoid formation. Osteoclasts are multinucleated cells that resorb bone through enzymatic digestion. Osteoblasts entrapped in the bone matrix, morph into osteocytes, which communicate with each other and the bone surface through cytoplasmic projections. Nonremodeling bone surfaces are covered by lining cells.

as much as 95% of all bone cells. Osteocytes are thought to be mechanosensors, meaning that they can perceive changes in tissue stress to ultimately remodel bone mass, and may coordinate the remodeling process carried out by both osteoblasts and osteoclasts. While the question of how osteocytes differ from osteoblasts at the gene expression level has been an important one, the technical limitations of in vitro assays, the lack of appropriate cell surface markers, or the inability to purify large quantities of homogenous population of cells have prevented a global analysis of their entire transcriptome. It was not until the generation of transgenic mice carrying fluorescent markers expressed by osteoblast lineage directed promoters that relatively homogeneous populations of osteoblasts and osteocytes were isolated and the gene expression profiles of these cells were examined.[15]

To purify osteoblasts and osteocytes from animal bone, Paic et al.[15] utilized two transgenic mice: one in which a 2.3 kilobase (kb) *collagen type I* promoter fragment directs the expression of the cyan variant of green fluorescent protein (GFP) to mature osteoblast lineage cells [$Col2.3^{Cyan}$ (blue)], and one in which a 7.9 kb region of the *Dmp1* promoter, along with a 4.4 kb region containing the first exon, the entire 1st intron, and a small part of the 2nd exon, to direct GFP topaz expression in osteocytes [$Dmp1^{Topaz}$ (yellow)]. Prior to cell purification, the authors evaluated cell-type specificity through histological evaluation of reporter expression in calvaria of double transgenic neonatal mice. They detected strong $Col2.3^{Cyan}$ reporter expression within the osteoblasts lining the bone surfaces and in some, but not all, osteocytes. In contrast $Dmp1^{Topaz}$ expression was found exclusively in cells fully or partially embedded in the bone matrix, highlighting the ability of this method to discriminate between osteoblasts and osteocytes in neonatal calvarial bone based on specific GFP expression. Pools of calvaria obtained from double transgenic GFP-reporter mice were subjected to sequential enzymatic digestion regimes followed by FACS sorting to facilitate the enrichment of $Col2.3^{Cyan+}/Dmp1^{Topaz-}$ and $Dmp1^{Topaz+}$ (both positive and negative for $Col2.3^{Cyan+}$) cells designated as highly enriched osteoblast and osteocyte/preosteocyte fractions, respectively. The global gene expression profiles of three sorted cell populations: GFP negative, $Col2.3^{Cyan+}$ (osteoblasts), and $Dmp1^{Topaz+}$ (preosteocytes and osteocytes) were analyzed using the MouseWG-6 v1BeadChip Illumina expression platform. To determine the comprehensive gene expression pattern behind the process of osteoblast-to-osteocyte transformation they also compared gene expression profiles of fluorescence-sorted, ($Col2.3^{Cyan+}$) versus ($Dmp1^{Topaz+}$) cell populations. Of the 3444 genes found, to be expressed in all 3 populations, 385 genes were differentially expressed between $Col2.3^{Cyan+}$and $Dmp1^{Topaz+}$ cell populations (of which 249 were ≥2-fold changed and 136 were ≤0.5-fold changed in the $Dmp1^{Topaz+}$ population).

One category of genes (87 genes; 66 low/21 high in osteocytes) found to be differentially expressed between osteoblasts and osteocytes included genes encoding bone abundant extracellular proteins typically secreted by osteoblasts.[15] Many of these genes encode collagen proteins (*Col15a1*, *Col18a1*, *Col4a1*, *Col22a1*, *and Col4a2* high in osteocytes; *Col16a1*, *Col27a1*, *Col3a1*, *Col9a2*, *Col9a1*, *Col8a2*, *Col12a1*, *Col14a1*, *Col2a1*, and *Col8a1* high in osteoblasts). Various metalloproteinases (*Mmp9*, *Mmp23*, *Adamts18*) exhibited less expression in osteocytes along with other genes with peptidase activity: reelin (*Reln*), tubulointerstitial nephritis antigenlike gene (*Tingal*), Htra serine peptidase 1 (*Htra1*), serine protease 12 (*Prss12*), metallocarboxypeptidase CPX-1 (*Cpxm1*), and complement factor (*Cfb*); *Reln* and *Tingal* peptidases had higher expression in osteocytes.[15] Members of the proteoglycan family, such as: keratocan (*Kera*), aggrecan 1 (*Acan*), proteoglycan 4 (*Prg4*), hyaluronan, proteoglycan link protein 1 (*Hapln1*), asporin (*Aspn*), chondroadherin (*Chad*), and fibromodulin (*Fmod*), were also downregulated in osteocytes. Other noncollagenous structural components normally present in the extracellular matrix of bone and cartilage were also dramatically reduced in osteocytes; these included fibulins (*Fbln1*, *Fbln2*), thrombospondins (*Thbs2*, *Thbs3*), spondin 1 (*Spon1*), cartilage oligomeric matrix protein (*Comp*), proline arginine-rich and leucine-rich repeat protein (*Prelp*), and matrilin 1 (*Matn1*).[15]

Several other genes previously identified as contributors to bone-related functions were found to be differentially expressed between osteoblasts and osteocytes.[15] Dentin matrix protein 1 (*Dmp1*), as expected, was 7.3-fold enriched in osteocytes, while periostin (*Postn*) was upregulated in osteoblasts. Several members of the transforming growth factor beta (*TGF-βs*), bone

morphogenetic proteins (*Bmps*), insulin-like growth factors (*Igfs*), and fibroblast growth factor families (*Fgfs*) were also found differentially expressed between these two related cell types. Strikingly, BMP signaling ligands *Bmp4*, *Bmp8a*, and *Bmp3* and WNT signaling antagonist *Dkk1* were more than twofold upregulated in osteocytes, while WNT signaling ligands *Wnt9a*, *Wnt10a*, and WNT antagonist *Dkk3* were more than twofold enriched in osteoblasts. Several other growth factors, such as glial cell line-derived neurotrophic factor (*Gdnf*), growth differentiation factor 10 (*Gdf10*), and pleiotrophin (*Ptn*) had lower levels in osteocytes. In particular, WNT signaling has recently emerged as a major regulator of bone formation and bone mass where loss of function mutations in the low-density lipoprotein receptor-related protein 5 (*LRP5*) cause low bone mass (LBM) in humans and mice,[51,52] while certain single amino-acid substitutions (e.g., G171V) in *LRP5* cause high bone mass (HBM). In addition, regulating WNT signaling at the receptor level, via protein antagonists, such as sclerostin (*Sost*) or Dickkopf homolog 1 (*Dkk1*) generate complementary phenotypes in mice (*Sost* knock-outs have HBM[53] and *Sost* transgenics have LBM) and humans (sclerosteosis[54]). While generally *Sost* has been described as an "osteocyte-specific negative regulator of bone formation" and is accepted as a marker of osteocyte function and activity, the authors of the osteoblast/osteocyte comparison did not find *Sost* among the transcripts significantly upregulated in osteocytes.[55] As an explanation for this observation they suggested that *Sost* may be regulated differently in neonatal calvaria, hence their samples may have lower levels of *Sost* expression than the expression in cortical osteocytes of adult mice.

Significant transcriptional differences were also observed between osteoblasts and osteocytes among 76 genes encoding plasma membrane associated proteins (52 genes ≥twofold in osteocytes), including *Phex*, *Ank*, *Gnas*, *Enpp1*, as well as *Pdpn* (podoplanin), which is known as the earliest osteocyte-selective protein expressed during osteoblast–osteocyte differentiation. Several members of the Notch signaling were also upregulated in osteocytes, including *Notch1*, *Notch3*, and *Dlk1*. Interestingly several FGF receptors were highly expressed in osteoblasts, but not in osteocytes, including *Fgfr2* and *Fgfr3*. Among known transcription factors, 31 were differentially expressed, 23 of which were ≥twofold enriched in osteocytes. These included: *Hey1*, *Dlx3*, *Pitx2*, *Tbx3*, *Irx5*, and *Irx6*. The group of genes with high expression in osteoblasts included *Zic1*, *Gsc*, *Runx1t1*, *Pax1*, and *Scx* a transcription factor with a basic helix-loop-helix motif known to function in tendons[56] and developing cartilage.[57] Some unexpected osteocyte-enriched candidates included genes previously shown to regulate muscle function, such as transcription factors *Myocd* and *Smyd*; structural myofibril components, such as *Myh11*, *Csrp3*, *Sync*, *Acta1*, *Dmd*, *Ttn*, *Tcap*, *Tnnt2*, *Tnni1*, *Myoz2*, *Tnnt3*, *Atp2a1*, *Tnnc2*, *Pdlim3*, *Tnnt1*, *Actn2*, *Tpm2*; and genes involved in myogenesis, such as *Casq2*, *Atp2a2*, *Gucy1a3*, *Atp1a2*, *Srpk3*, and *Csrp3*. In particular, one surprising discovery was that skeletal muscle actin alpha 1 (*Acta1*), skeletal muscle protein troponin 2 (*Tnni2*), fast skeletal muscle myosin light chain (*Mylpf*), troponin C2 (*TnnC2*), myosin heavy polypeptide 11 (*Myh11*), and myosin light polypeptide 1 (*Myl1*) all topped the upregulated gene list with ≥20-fold upregulation in osteocytes.

Thus, the approach presented by Paic et al.[15] is unique in bone biology and the data generated has the potential to highlight new important regulators of both osteoblast and osteocyte function. In particular, the role of myogenic genes is intriguing since recently it has been reported that PTH mediates *Sost* expression via an unlikely candidate: the *Mef2C* transcription factor, a well-established regulator of cardiac and muscle development.[58] It is however important to highlight several caveats which may limit the generalization based on the experimental design of Paic et al.[15] of the "osteocyte" and "osteoblast" transcriptome to adult bone function. These include: (1) the age of the purified cells which were derived from neonatal bone that may differ from cells obtained from the adult skeleton; (2) the anatomical location of the cells collected: *calvaria* versus *long bones*; and (3) the potential for intrinsic differences in mechanosensitivity of cells derived from *axial* versus *appendicular* bones. In the Paic et al.[15] study, these cells were obtained from neonatal mice when the organism is undergoing rapid growth and development. The bone therefore is metabolically very active and the balance of bone formation is in a "modeling" phase, with relatively very little remodeling. As an organism reaches maturity, bone formation slows down, and bone metabolism is driven by a balance between formation and resorption characteristic of bone remodeling. Therefore, from a metabolic point of view, the osteoblasts and osteocytes may display different "transcriptional profiles" as a function of age. In addition, there are distinct differences between bone patterning that occurs in intramembranous versus endochondral bone formation and subsequent ossification. Accordingly, it is prudent to assume that osteoblasts and osteocytes derived from the axial skeleton may differ molecularly from those derived from the appendicular skeleton. Another source of molecular difference may stem from how cells interpret mechanical forces. While osteocytes and osteoblasts are mechanosensitive, it is very likely that the biophysical forces perceived by resident cells within the calvaria are fundamentally different than in long bones. While some of the candidate genes highlighted by this study may overlap with genes that control biomechanical function in loaded long bones, this represents a hypothesis and requires further validation.

Osteoclast differentiation is a complex process that takes roughly 4 days in vivo, during which mononuclear precursor cells migrate to the site of resorption, fuse to form multinucleated osteoclasts, form specialized structures including actin ring and ruffled borders, and attach firmly to bone, sealing them to the bone surface. Subsequently, these osteoclasts secrete protons to demineralize the bone and proteases to degrade the proteinaceous extracellular matrix. Gene expression studies using bone marrow precursors[59–63] or RAW264 macrophage cell line[64–65] differentiating toward osteoclasts in vitro, have significantly enhanced our understanding of osteoclastogenesis. Notably, Takayanagi et al.[60] and Ishida et al.,[64] using microarrays, identified several genes differentially regulated during osteoclastogenesis including NFATC-1which was subsequently validated in animal models and identified as a key regulator of osteoclast differentiation.[66,67] Together, these studies further our knowledge of genomic changes during osteoclastogenesis. However, more in vivo functional studies are required to determine the precise functions of these genes in osteoclastogenesis.

1.2 Profiling Location and Age Dependent Changes in Skeletal Gene Expression

Numerous studies have identified several differences in the adult bones derived from distinct primary ossification processes including differences in matrix composition, mineral density, and calcium concentration. It has also been shown that the skull bone responds differently to mechanical strain compared to limb bones; furthermore osteocytes of mouse calvaria have a more spherical shape compared to the more elongated osteocytes of long bone.[16] It is therefore reasonable to hypothesize that there is a difference in the transcriptome of bone cells collected from different anatomical locations, reflecting their specific functions and origin. To address this question, using Affymetrix microarrays, Rawlinson et al. profiled the transcriptome of rat ulnar limb and parietal skull bone, and osteoblasts derived from skull and limb bones.[16] For the majority of genes, calvaria and ulna showed very similar expression patterns confirming a high degree of similarity among bone tissues, irrespective of their origin and location. However, they identified 1236 genes significantly differentially expressed between skull and long bones. Several genes known to be associated with bone development including *Opg*, *Pthr1*, *Calcr*, *Lrp5*, *Sost*, *Ibsp*, *Bmp3*, *Bmp6*, *Cthrc1*, *Ptn*, and *Ctsk* were upregulated in skull bone compared to long bone while genes, such as *Wnt16*, *β-catenin*, *Bmp5*, and *Comp* showed an elevated expression in long bones. Rawlinson et al. also identified several transcription factors differentially expressed between these anatomically distinct bone locations, which may account for their site-specific properties. Transcription factors including *Sp7*, *Vdr*, *Tcf7*, *Fos*, *Dlx5*, *Dlx3*, *Tbx2*, and *Twist1* were found to be upregulated in calvarial bone whereas transcription factors *Sox6*, *Gata1*, *Gata3*, *Cited4*, and several members of Hox family including *Hoxa5*, *Hoxa7*, *Hoxa10*, and *Hoxc5* were found to be upregulated in long bone. They also observed that the number of osteocytes per unit volume was significantly higher in limb bone compared with skull bone.

Rawlinson et al. found that majority of the differences identified between skull and limb bone transcriptome profiles were lost in cultured osteoblasts. However, they identified 249 genes significantly differentially expressed between skull and limb bone-derived osteoblasts, in vitro. Consistent with the regional differences observed in whole bone gene expression profiles, they detected an elevated expression of Hoxa cluster genes (*Hoxa1*, *Hoxa4*, *Hoxa5*, *Hoxa7*, *Hoxa10*, *Hoxa13*) in limb derived osteoblasts compared to skull derived osteoblasts, which was further confirmed by quantitative PCR (qPCR). Genes upregulated in skull derived osteoblasts include *Sfrp2*, *Apoe*, *Irx5*, and *Msx2*. These findings suggest that at least some of the regional changes in gene expression are independent of local microenvironment but represent an inherent property of these cells.

In a subsequent study, Kingsmill et al. compared the gene expression profiles of adult rat mandible to ulna and calvaria.[17] Approximately 84% (23,232) of the transcripts analyzed had similar expression levels in the three functionally distinct skeletal locations. They also identified 1850 transcripts unique to a single site whereas 1014 transcripts were found to be common only to mandible and calvaria, and 873 transcripts were common only to mandible and ulna. Genes highly expressed in mandible and calvaria, but not in the ulna included *Fos*, *Gli1*, *Gli2*, *Tcf7*, *Bmpr2*, and *Smad3*. Genes highly expressed in mandible and ulna, but not in the calvaria included *Pax6*, *Cited4*, *Cdh1*, *Shox2*, and *Tnf*. These two studies support the hypothesis that genomic profiles of osteoblasts differ based on their anatomical location and suggest that these differences are established, at least in part, by the developmental origin and history of the cells at different anatomical sites.

While Rawlinson et al. and Kingsmill et al. investigated how the gene expression profiles of bones from different skeletal sites differ in relation to their origin and location, Farr et al.[18] studied the transcriptional changes in bone as a function of age. Farr et al. obtained needle bone biopsies (1–2 mm diameter) from the posterior iliac crest of 20 young women (mean age ± SD, 30.0 ± 5.4 years, range 22–40 years) and 20 old women (72.9 ± 6.5 years, 65–88 years) and profiled the transcriptome using RNAseq. This study identified 678 genes (446 up- and 232 downregulated genes) with altered expression in old relative to young women.

Expression levels of several genes from the Notch pathway including *NOTCH3*, *NOTCH4*, *JAG2*, *DLL4*, *HES1*, and *HEY1* were significantly higher in bone biopsies of old versus young women. They also observed alterations in the expression of several WNT-signaling pathway members. WNT pathway members *SFRP5* and *LEF1* were found to be decreased, whereas levels for *FZD4*, *SFRP1*, and *CCND1* were found to be increased in bone biopsies of old women. However, there were no changes in the expression of *SOST*, *LRPs* (*LRP4-6*), and *DKKs*, WNT family members previously linked to bone metabolism. They also identified several genes from eNOS signaling pathway, PDGF signaling, and Oncostatin M signaling with altered expression in old versus young subjects.

Farr et al. also profiled the gene expression changes induced by estrogen therapy in old subjects and identified several genes including *LGR5*, *PPARGC1A*, *INHBB*, and *C19orf80* with altered expression in response to estrogen. Estrogen therapy restored the expression of *INHBB*, which was significantly decreased with aging, to young levels, suggesting that alterations in estrogen levels might be responsible for some of the changes observed between young and old women. However, estrogen therapy only changed the expression of 21 genes of the 678 genes altered with age. These studies suggest that genes associated with bone mass and mineral density are differentially expressed in functionally distinct skeletal sites,[16,17] and also as a function of age.[18,68] These spatial and temporal differences in gene expression must be taken into account when generalizing the results from different gene expression studies.

1.3 Profiling Biomechanical Effects on Bone

Mechanical stimuli, such as vibration, axial loading, and bending regulate bone shape and strength, activate bone formation, and increase BMD.[69] To identify genes and signal pathways responsible for mechanical loading-induced bone formation, Zhang et al.[19] examined the global gene expression changes resulting from joint loading.[19] Joint loading induces an anabolic response and the authors were interested to determine if ankle loading promotes bone formation in the tibia. Accordingly, the left ankle of 14-week-old C57BL/6 mice was loaded 3 min/day for 3 consecutive days (0.5 N/5 Hz). Bone RNA was collected from the right (control) and left (loaded) tibia at 1 h after the 3rd loading and processed using Agilent microarrays. Using histomorphometry the authors determined that there was a corresponding 10% increase in cross-sectional cortical bone area in response to loading. The microarray comparison identified 242 transcripts upregulated ≥1.2-fold and 199 transcripts downregulated ≤ 0.8-fold in response to ankle loading. A more stringent comparison of genes that changed more than twofold from loading identified a group of 50 transcripts including *Mmp3*, *Has*, *Mrgpra2a*, *Thbs3*, *Timp1*, *Col3a1*, *Matn2*, *Matn4*, *Cd248*, *Cspg4*, *Fosb*, *Aspn*, *Cdh13*, *Aqp1*, *Moxd1*, *Anxa8*, *Atf3*, *Aebp1*, *Prrx2*, *Cma1*, *Hapln1*, *Il1rl1* (upregulated); and *Btc*, *Senp6*, *Lrrc34*, *Barhl2*, *Tnfp*, *Phf20l1*, *Kirrel3*, *Crisp1*, *Npy2r*, *Txnax*, *Krt82*, *Ptgs2*, *Pax7a*, *Znf236*, *Crlf1* (downregulated).[19]

In a more recent study, Mantila Roosa et al.[20] evaluated loading-induced gene expression in rat ulna over a time course of 4 h to 32 days (d) (4 h, 12 h, 1 d, 2d, 4d, 6d, 8d, 12d, 16d, 24d, and 32d). Using Affymetrix microarrays they identified 1051 genes that were differentially expressed in at least one time point, in response to loading. After performing a gene expression clustering they identified six distinct patterns of differential gene expression: an early-response cluster in which the genes were upregulated early but not late in the time course, three matrix-formation (up) clusters that followed the pattern of matrix synthesis, and two matrix-formation (down) clusters that were downregulated during matrix formation. Several chemokines (*Ccl2*, *Ccl7*, *Cxcl1*, *Cxcl13*), calcium signaling genes (*Anxa2*, *S100a4*, *S100a10*), matrix proteins (*Adamts1*, *Ecm1*, *Serpina3n*, *Serpine1*, *Tfpi2*), and AP-1 transcription factors (*Fosl1*, *Junb*) were identified as upregulated early on, primarily at 4 h. Mantila Roosa et al. also identified several extracellular matrix genes (upregulated: *Alpl*, *Bglap*, *Col1a2*, *Cthrc1*, *Fn1*, *Ibsp*, *Lox*, *Sparc*, *Vcan*, *Bgn*, etc.; downregulated: *Efemp1*, *Mmp8*, *Prelp*, *Serpinb2*, *Spon1*) and growth factors (upregulated: *Fgf14*, *Pdgfa*, *Pdgfc*, *Pdgfrl*, *Pgf*; downregulated: *Egf*, *Fgf1*, *Fgf7*, *Fgf23*, *Fgl2*, *Hgf*) as differentially regulated during matrix formation, in response to loading. Several ion channels and solute carriers were also differentially regulated during matrix formation. Interestingly, several muscle-related genes were identified as downregulated in loaded ulna (*Acta1*, *Dmd*, *Myocd*, *Myl1*, *Myplf*, *Tnni2*, *Tnnt3*, *Tpm2*), genes that were also identified as enriched in osteocytes compared to osteoblasts by Paic et al. Other important bone metabolism genes differentially regulated during matrix formation included *Vdr*, *Tgfb1*, *Tgfb3*, *Bmp2*, *Wif1*, *Wisp1*, *Pthr1*, *Sp7*, *Jund* (upregulated genes), *Grem1*, *Bmpr1b*, *Tgfbr3*, *Chrdl1*, and WNT pathway inhibitors *Sost* and *Sfrp4* (downregulated genes).

While studies by Zhang et al.[19] and Mantila Roosa et al.[20] identified changes in whole bone transcriptome in response to loading, Wasserman et al.[21] studied transcriptional changes specifically in trabecular osteocytes from mouse vertebra subjected to controlled compression loading. Using Affymetrix microarrays they profiled gene expression changes in osteocytes isolated from vertebrae receiving a single 8 N loading dose or repetitive loading compared to osteocytes from sham-loaded

vertebral trabecular bone. By this approach, Wasserman et al. identified 287 up- and 52 downregulated genes in response to a single load in osteocytes. Upregulated genes included *Wnt5a*, *Aspn*, *Igf1*, *Emilin2*, *Ccl12*, *Adamts4*, and *Adamts1* whereas *Olfm3* and *Spock2* were identified as downregulated. 1339 genes were differentially regulated (778 up- and 561 downregulated) in osteocytes isolated from vertebra subjected to repetitive loading. Genes upregulated by repetitive loading included *Ptn*, *Vcan*, *Aspn*, *Thbs4*, *Sema3c*, *Sema3d*, *Thbs2*, *Thbs1*, *Mmp2*, *Cyr61*, *Grem1*, *Fgf18*, *Grem2*, *Pdgfd*, *Wnt5a*, and *Dmp1* whereas *Ltbp1*, *Muc4*, and *Apoa5* were downregulated. In summary, Wasserman et al. identified several genes, in particular those encoding extracellular proteins, differentially regulated in osteocytes in response to loading. It is interesting to note that *Sost* was not detected among the significantly downregulated transcripts though it is generally recognized that mechanical loading downregulates *Sost* expression in osteocytes.[70,71]

Multiple studies have suggested that mechanical loading differentially increases bone mass in cortical and cancellous sites. In a recent study, Kelly et al.[22] investigated transcriptional changes in cortical and cancellous bone in response to mechanical loading. In this study the left tibia of 10-week-old C57Bl/6 female mice was subjected to a single load and cortical and cancellous bone was collected 3 and 24 h after loading and changes in gene expression were profiled using RNAseq. At 3 h postloading, 43 and 18 genes were found differentially expressed in loaded cortical and cancellous bones, respectively. Genes upregulated in both cortical and cancellous bone included *Wnt1*, *Wnt7b*, *Timp1*, *Ptgs2*, and *Opg*. Enriched signaling pathways included WNT signaling in both cortical and cancellous bone and hedgehog signaling in cortical bone only. Apart from *Wnt1* and *Wnt7b*, an increased expression of *Wnt10b* was also observed at 3 h after a single loading session, in the cortical bone, while *Lrp5* was modestly decreased. WNT inhibitors *Sost* and *Dkk1* were found to be downregulated in cancellous bone 3 h after loading, but were not significantly changed in the cortical bone. At 24 h postloading, 58 genes were differentially expressed in cortical and 32 genes in cancellous bone. 12 genes (*Ptn*, *Vcan*, *Cthrc1*, *Hapln4*, *Tubb3*, *etc.*) were commonly changed in both cortical and cancellous bone. *Wnt1* and *Wnt10b* remained upregulated at 24 h postloading in cortical bone. Interestingly, several muscle-related genes (*Myh4*, *Tnnc2*, *Smpx*, *Tnnt3*, *Mylk2*, *Myoz1*, *Myom2*, *Actn3*, *Myl1*, *Casq1*, *Mybpc2*, *Myh2*, *Mylpf*, *Tnni2*) were found to be downregulated in cancellous bone at 24 h postloading. It is important to note that several of these muscle-related genes including *Tnnc2* and *Tnnt3* were also identified by Paic et al. as enriched in osteocytes and by Mantila Roosa et al. as downregulated in the loaded ulna. Kelly et al. also observed that *Wnt16*, *Wnt7b*, and *Sost* were highly expressed in unloaded cortical bone compared to unloaded cancellous bone. High expression of *Wnt16* in cortical bone is consistent with the cortical bone phenotype in mice lacking *Wnt16*.[72] Other genes highly expressed in unloaded cortical bone include bone metabolism related genes *Postn*, *Aspn*, *Mepe*, *Ostn*, and *Ptn* while genes including *Grem1*, *Kcnj5*, and *Slc24a5* showed high expression in unloaded cancellous bone. This study highlights the importance of WNT signaling in load induced bone formation and provides novel insights into differential response of cortical and cancellous bone to mechanical loading. Further investigation into the downregulation of muscle-related genes in cancellous bone is required to understand the functions of these genes in bone.

Tissues derived from an organism are highly heterogeneous and the transcriptional profile represents a comprehensive sum of multiple cell-type outputs. A highly specialized signal that comes exclusively from one cell type may be diluted in tissue samples if the surrounding tissues may exhibit the opposite transcriptional effect. Wasserman et al.[21] purified osteocytes from loaded bone in an attempt to understand the changes specific to this cell type and identified several differentially regulated genes. However, they failed to recapitulate several known load induced changes in osteocyte transcriptome including downregulation of *Sost*. Studies by Mantila Roosa et al.[20] and Kelly et al.[22] highlighted the importance of studying loaded induced changes as a function of time; they observed significant differences in the loaded bone transcriptome at different time points. Future studies will have to rely on purifying homogenous populations of cells, similar to the study described in the first section by Paic et al.,[15] from different time points in order to distinguish cell autonomous from noncell autonomous effects and to understand temporal changes.

1.4 Profiling Gene Expression Changes Occurring as a Function of Altered Bone Metabolism

1.4.1 Profiling Bone in Animal Models With Impaired Bone Metabolism

In this section we will discuss recent work that describes global gene expression changes that occur as a function of altered bone metabolism (resulting in net gain or loss of bone mass) in animal models. OP has been linked to age-related postmenopausal hormonal changes in women, and is strongly correlated with decreases in estrogen-related bone signaling therefore, ovariectomized (OVX) mice and rats represent an established animal model for postmenopausal bone loss.[73] Xiao et al.[23] used this OVX animal model to compare the genomic

profile between OP, non-OP, aged, and juvenile rats.[23] They applied microarrays with 34,000 known genes to examine gene expression in bone marrow mesenchymal stromal cells isolated from the bone marrow of the four different treatment groups. Comparisons between OP (7-month-old OVX) and non-OP (7-month-old) identified 195 up- and 109 downregulated transcripts; non-OP versus aged rats (>2-years old) identified 62 up- and 86 downregulated transcripts; and young (7-weeks old) versus older(7-month-old), non-OP rats identified 120 up- and 80 downregulated genes. OP caused an increase in the expression of cell adhesion-related gene *Spon1*, adipogenic differentiation genes (*Alox5*, *Baat*, *Sult4a1*, *Lpl*), and genes involved in cell growth and maintenance (*Mmp8*, *Cyp3a9*, *A2m*, *Alpl*, *Crabp2*, *Slc26a1*, *Cdkn2b*). Many genes were also found to be downregulated in OP, such as *Npy* (neuropeptide Y, involved in bone growth), *Cd24* (promotes proliferation and differentiation), *Ramp3* (osteoclast regulator), *Marcksl1* (involved in calcium channel function), *Wnt4*, *Plpcb* (prolactin-like protein C beta; reported to have inhibitory effects on osteoblast formation), and *Adrb3* (glucose and lipid metabolism). In addition, this paper compared genes that were upregulated in OP versus aged tissue, and found that there were 14 genes upregulated in OP and downregulated in aged rats (*Mmp8*, *Sponf1*, *Csrp2*, *Ivl*, *Folrl*, *Crabp2*, *Loc64305*, *Nac1*, *Gludins*, *Braf*, *Inhbp*, *Pgr*, *Slc26a1*, *Sp1*) and 6 genes downregulated in OP but upregulated in aged rats (*Prlpb*, *Iilrn*, *Plpcb*, *Loc171569*, *Ramp3*, *Mip*).

Runx2 has been shown to play a key role during skeletogenesis.[74] In *Runx2* knockout mice both intramembranous and endochondral bone formation is blocked. These mice lack mature osteoblasts and have a skeleton made of cartilage.[74] To identify *Runx2* target genes, Vaes et al.[24] compared the gene expression of skeletal tissues (calvaria, forelimbs, and hindlimbs) in *Runx2* knockout mouse embryos at 14.5 dpc to wild-type controls. The authors found 1277/22,625 transcripts (531 downregulated in KO), 606 (296 downregulated in KO), and 492 (172 downregulated in KO) to be differentially expressed in calvaria, forelimb, and hindlimb, respectively. These data suggested that loss of the *Runx2* transcription factor had both positive and negative effects on gene expression in skeletal tissue.[24] To identify potential *Runx2* targets, the authors searched for transcripts that were modulated similarly in all three tissues. They found 41 transcripts (35 known genes and 6 ESTs) to be upregulated and 25 transcripts (22 genes and 3 ESTs) to be downregulated in all three datasets including: *Ibsp*, *Spp1*, *Akp2*, *Runx2*, *Dlx5*, *Matn4*, *Mmp9*, *Col9a1*, *Scgf*, *Agc1*, as well as 15 genes previously unknown to be *Runx2* targets or to function during bone formation: *Pim1*, *Satb2*, *Snf1lk*, *Cfh*, *Gtpbp2*, *Tubb5*, *Hck*, *H2-T10*, *Tce1*, *Ppp2r5d*, *Ndufb10*, *Fdps*. The other three novel transcripts were ESTs or Riken cDNA clones.[24]

Two recently published papers have taken a novel approach for studying global gene expression changes.[25,26] They used genes identified from human linkage studies, eQTL analysis, high-density SNP maps, causality modeling, and genome-wide association studies and integrated them with genetic variants, gene expression profiling, and skeletal phenotypes in inbred mice to help identify candidate genes for bone phenotypes.[25,26] These approaches are very complex and should be read in detail; however a number of important genes were identified as being strongly correlated with OP. Genes highlighted by Farber et al.[26] include *Grem2*, *Twist2*, *Ccdc28b*, *Timd4*, *and Smad4* with additional emphasis on *Rasd1* and *Wnt9a* (the two most significantly related genes and thus the two strongest candidates for OP delineated in this paper). RAS, dexamethasone-induced 1 (*Rasd1*) is a Ras family GTPase thought to be involved in circadian rhythm.[75] *Wnt9a*, on the other hand, is a member of the canonical WNT signaling pathway, and is involved in joint formation and maintenance in addition to being linked to skeletal formation and growth.[76] Using a similar integration approach, Hsu et al.[25] identified 16 candidate genes, *Ppap2b*, *Gpr177*, *Hecw2*, *Casr*, *Mmrn1*, *Irx2*, *Pdzd2*, *Tgfb1*, *Cacnb2*, *Dock1*, *Sox6*, *Pdgfd*, *Rad51L1*, *Sall1*, *Fbxo31*, and *Cdh2*. Of note, *Ppap2b* and *Gpr177* have both been previously shown to be involved in the regulation of the WNT receptor-signaling pathway; *Ppap2b* via a glycerolipid and glycerophospholipid metabolism pathway and *Gpr177* through regulation of I-kappB kinase/NF-kappaB cascade pathway.[25,77] In addition, *Sox6* and *Cdh2* have also been linked to the canonical WNT/β-catenin signaling pathway.[78,79] Furthermore, *Casr*, the gene encoding the calcium-sensing receptor, is known to interact with *Vdr/Rxr* activation and is important in the mineralization of bone.[80] *Tgfβ1* and *Cacnb2* have also both been linked to endochondral bone formation in cartilage and calcium ion transportation, respectively, indicating their importance in bone ossification.[81,82]

Mutations in WNT coreceptor low-density lipoprotein receptor-related protein 5 (LRP5) cause high or LBM phenotypes depending on the nature of mutation. Ayturk et al.[27] profiled the transcriptome of *Lrp5* wild type ($Lrp5^{+/+}$), *Lrp5* knockout ($Lrp5^{-/-}$), and HBM-causing ($Lrp5^{p.A214V/+}$) knock-in alleles to identify genes and molecular mechanisms altered in these genotypes. Using RNAseq they identified 302 genes differentially expressed between $Lrp5^{-/-}$ and $Lrp5^{+/+}$, 166 genes differentially expressed between $Lrp5^{-/-}$ and $Lrp5^{p.A214V/+}$, and 28 genes differentially expressed between $Lrp5^{p.A214V/+}$ and $Lrp5^{+/+}$ in mouse diaphyseal bone. A large number of genes downregulated in $Lrp5^{-/-}$ compared to $Lrp5^{+/+}$ mice, including *Col1a1*, *Col1a2*, *Bglap*, *Mt1*, *Mt2*, *Cyr61*, *Cgref1*, and *Gata1*, overlapped with genes downregulated in $Lrp5^{-/-}$ compared to $Lrp5^{p.A214V/+}$ mice. Interestingly, two positive regulators of WNT pathway, *Wnt10b*

and *Fzd4*, exhibited significantly increased expression in $Lrp5^{-/-}$ compared to $Lrp5^{p.A214V/+}$ and $Lrp5^{+/+}$ mice. Other genes upregulated in $Lrp5^{-/-}$ compared to $Lrp5^{p.A214V/+}$ and $Lrp5^{+/+}$ included *Postn*, *Thbs2*, and *Mdm4*. Another interesting finding was the upregulation of *Wnt16*, a positive regulator of bone mass, in $Lrp5^{-/-}$ compared to $Lrp5^{+/+}$. Genes differentially expressed in $Lrp5^{p.A214V/+}$ compared to $Lrp5^{+/+}$ included *Kera*, *Myoc*, *Angptl7*, *Serpina1a*, *Serpina1d*, *Serpina1b*, *Serpina1e* (upregulated), *Igkv9-123*, *Igkv1-88*, *Igkv8-27*, and *Ighv7-1* (downregulated). Further studies are required to understand the roles of these genes in LRP5-mediated regulation of bone mass.

Inhibition of WNT pathway inhibitor Sost with Sost antibody (Scl-Ab) has been shown to promote bone formation and decrease bone resorption, ultimately leading to an increase in bone mass in both human and animals. Nioi et al.,[28] using Affymetrix microarrays, profiled transcriptional response associated with Scl-Ab administration (single dose, 100 mg/kg) in mature osteoblast subpopulations [osteoblasts (OB), lining cells (LC), and osteocytes (OCy)] at several time points (6, 24, 72, and 168 h postdose), after enrichment with laser capture microdissection. The number of probe sets changed in response to Scl-Ab in any cell type or time point ranged from 0 to 514. Transcriptional changes caused by Scl-Ab were very similar across all three cell types. Majority of well-known canonical WNT signaling genes did not show significant change in their expression, in response to Scl-Ab; however, this study identified several WNT targets (*Wisp1*, *Twist1*, *Bglap*, *Gja1*, *Tcf7*, *Enpp2*, *Mmp2*) as Scl-Ab-responsive genes. Four genes, *Wisp1*, *Twist1*, *Bglap*, and *Mmp2* showed progressive upregulation across the time course and a similar pattern in all three cell populations after Scl-Ab administration. This study also identified several genes involved in synthesis and mineralization of bone matrix (*Omd*, *Bgn*, *Dcn*, *Sparc*, *Lepre*, *Lox*, *Bmp1*, *Alpl*) as differentially regulated in response to Scl-Ab. Other key genes regulated by Scl-Ab included *Tmem119*, *Pdgfa*, *Cxcl12*, *Cxcl14*, *Dlx5*, sphingolipid signaling genes (*Smpd*, *Sgms2*, *S1pr3*, *Spns2m*), and cell cycle genes (*Cdkn1a*, *Cgref1*, *Aurka*, *Bub1*, *Ccnd1*).

In another study, Taylor et al.[29] profiled time dependent transcriptional changes in OB, OCy, and LC in response to long-term Scl-Ab treatment (3 or 50 mg/kg/week) for up to 26 weeks (183 days), followed by a treatment-free period (TFP). The gene expression analysis was mainly focused on 50 mg/kg group where they profiled gene expression changes at days 8, 29, 85, 183, 237, and 309. This study identified several genes differentially regulated in response to chronic Scl-Ab administration, with greatest number of gene expression changes occurring in OCy and least changes in LC. Similar to Nioi et al., Taylor et al. also found that OB, LC, and OCy had very similar changes in their gene expression profiles at day 8, with an upregulation of a number of extra-cellular proteins and few canonical WNT targets including *Wisp1* and *Twist1*. However, on day 29, the WNT target gene expression pattern significantly changed in OCy with *Twist1* returning to vehicle levels and upregulation of a different set of WNT targets [*Cd44*, *Id2*, *Irx3*, *Vegfa*, *Tcf7l2* (*Tcf4*)] that remained elevated throughout the time course. Another set of WNT targets including *Birc5*, *Ccnd1*, and *Mmp9* showed a decrease in expression in response to Scl-Ab beginning on day 29. Changes in WNT signaling pathway were limited to upregulation of *Wnt5b* and WNT inhibitor *Sfrp4* in OCy. Scl-Ab treatment also upregulated cell cycle genes *Cdkn1a* and *Mdm2* in OCy at day 29 which remained elevated throughout the time course. Several other cell cycle genes including *Aurka*, *Bub1b*, *Chek1*, *Kif23*, *Kifc1*, *Knstrn*, and *Plk1* were downregulated beginning on day 29. Other important Scl-Ab induced genes included *Dmp1*, *Mepe*, *Phex*, *Postn*, *Tgfb3*, and *Bmp7* in OCy and *Col1a1*, *Alpl*, and *Mgp* in OB. Apart from the Scl-Ab responsive genes identified with microarrays, Taylor et al. also identified *Dkk1* and *Sost* as Scl-Ab inducible genes using TaqMan analysis.

Here we discussed some key findings from genomic profiling in animal models of OP; however, it is important to note that numerous other studies have also used microarrays or RNAseq to profile gene expression changes in animal models with altered bone metabolism.[83,84] Comprehensively, these animal studies have identified signaling pathways and groups of genes that act concordantly (many with previously unknown functions), suggesting that these cohorts of genes are functionally related during bone regulation. These results highlight the utility of animal models for delineating relevant biological pathways. However, animal models cannot fully replicate the complex genetic and environmental interactions that contribute to the development of OP and other bone-related diseases, in humans. Therefore, it is necessary to complement these animal models with studies of human tissues.

1.4.2 Profiling Osteoporosis in Humans

OP is actually a family of metabolic bone diseases defined using bone densitometry by a >2.5 standard of deviations below the average BMD value of a healthy, young person. OP can result in cortical bone thinning and porosity, fragility, and increased fracture risk.[85] Since adult bone mass homeostasis is achieved through a balance between bone formation and bone resorption, either an increase in bone resorption and/or a decrease in bone formation could lead to bone loss. Age, diet, physical activity, along with genetic predisposition, all contribute to these processes. Although there has been extensive progress in the use of genomics to identify genetic loci and variants that contribute to susceptibility to OP,[86–92] these polymorphisms account for very little of the overall variation in BMD. Therefore, many more

genes and genetic variants involved in the regulation of bone mass and susceptibility to skeletal disease await discovery. This section will address the current use of genomic expression profiling and its importance in understanding how genomic regulation results in complex bone disease.

Lei et al. compared circulating monocytes in premenopausal subjects ranging from extremely low peak bone mass (PBM) to high PBM.[30] Forty nine genes (37 upregulated and 12 downregulated) were differentially expressed between the two groups. Gene ontology biological process classification found 17 major categories including cytokine–cytokine receptor interaction, toll-like receptor signaling pathways, and Jak-STAT signaling pathways, all of which are significantly involved in bone metabolism and osteoclast differentiation. Following false-discovery rate adjustments, 3 of the 49 genes were found to be significant (*Stat1*, *Gbp1*, and *Cxcl10*). All three of these genes are known to be involved in osteoclastogenesis.[30,93,94] In a more recent study, Liu et al.[31] performed microarray analysis of monocytes in 42 (16 pre- and 26 postmenopausal) high hip BMD and 31 (15 pre- and 16 postmenopausal) low hip BMD Caucasian females. Their analysis identified several apoptosis genes including *DAXX*, *PLK3*, *PDCD5*, and *VDAC1* downregulated in low BMD females. Five genes associated with cell cycle arrest, *HBP1*, *CDKN2D*, *GAS2L1*, *PKD1*, and *PPP1R15A* were also down in low versus high BMD subjects. A metaanalysis of seven GWAS studies identified association of three of these genes, *VDAC1*, *DAXX*, and *PLK3*, with hip BMD. Two of the apoptosis induction genes *DAXX* and *PLK3*, both downregulated in the low BMD subjects, overlapped with previous studies from this group.[30,87] These findings suggest that decreased apoptosis of monocytes might be a contributing factor to low BMD.

Trost et al.[32] explored another approach for identifying OP-related genes by examining the transcriptional profile of cultured osteoblasts isolated from 55 unrelated patients undergoing hip arthroplasty due to osteoarthritis (non-OP) or femoral neck fracture (OP).[32] Eight postmenopausal (four with normal and four with low BMD) were also included in the study. Following data acquisition, microarray intensity values were compared to a predetermined set of genes thought to be characteristic of osteoblasts: *ALPl*, *BGLAP*, *BMP2*, *COL1A1*, *CSF1*, *LRP5*, *MGP*, *RUNX2*, *TNFRSF11b*, and *VDR*. Of these genes, bone matrix genes, such as *COL1A1, MGP*, and the *osteoprotegerin* gene, *TNFRSF11b*, had the highest expression levels in OP samples. In contrast bone differentiation factors *BMP2* and *RUNX2*, a matrix mineralization factor, *ALPL*, and the differentiation cytokine factor, *CSF1*, were dramatically reduced in the OP samples. In total, the microarray analysis detected 1606 genes differentially expressed, where 352 transcripts (144 upregulated; 208 downregulated) were changed by greater than 2.0-fold in the OP relative to the non-OP tissues. This paper indicated a large list of genes involved in OP, such as upregulated genes *CXCL2*, *ADRA2A*, *TSPAN8*, *SLC7A11*, *CNND2*, *PSG11*, and *COL15A1* and downregulated genes *PTN*, *IBSB*, *PRPF19*, *COL10A1*, *NGEF*, *CCNB2*, *CDC2*, and *PBK*.

Sorting the differentially expressed genes into KEGG canonical pathways identified ribosomal proteins (*Rpl13a*, *21*, *26*, etc.) and proteins involved in the cell cycle (*CCND2*, *CDKN1a*, *CDC2*, *CDNB2*, *CCNA2*, *CDK2*, etc.) as important pathways, suggesting that osteoblasts during OP differ both in their rate of proliferation and protein synthesis. Confirming this observation, Giner and coworkers[95] previously reported that osteoblasts from OP patients do in fact have decreased proliferative potential.[95] Other interesting pathways found included: cytokine–cytokine receptor interaction (*CCL2*, *CXCL2*, *CSF1*, *TNFRSF19*, etc.), regulation of actin cytoskeleton (*ARPC1A*, *ARPC1B*, *PPP1R12A*, *ACTN1*, *THOA*, *ARPC5*, etc.), and WNT signaling (*SFRP2*, *CCND2*, *CSNK2A2*, *PPP2CA*, *CTBP2*, *TCF7*, *PRICKl1*, *RHOA*, and *FZD5*). Therefore, this study showed the strength of microarrays in genomic profiling. Not only did the authors find that osteoporotic osteoblasts had upregulation of cytokine signaling and downregulation of the growth factor *PTN*, but this study also identified a list of potential candidate osteoporotic-genes previously uncharacterized in bone disease. It is also important to note that the *PTN* gene was also identified by Paic et al.[15] as an osteoblast gene that is dramatically repressed in osteocytes, while Wasserman et al.[21] and Kelly et al.[22] found *PTN* to be dramatically upregulated upon biomechanical stimulation. These results suggest that *PTN* deserves additional attention as a contributor to bone formation, mass maintenance, and biomechanical response. Further characterization of some of the genes highlighted by various microarray experiments may reveal them as potent therapeutic targets for the treatment of OP-related bone disorders.

Due to the difficulties of obtaining human tissue samples and the complexities of designing human experiments (identifying large cohorts matched for age, ethnicity, gender, etc.), very few microarray studies have used human bone. Hopwood et al.[33] used microarray analysis to identify candidate OP genes in trabecular bone from the intertrochanteric region of the proximal femur from 10 women undergoing hip arthroplasty for a fractured neck of the femur (OP), and 10 women undergoing hip arthroplasty for primary OA (non-OP) in addition to 10 women at autopsy (control). They found 150 transcript levels were changed in OP bone, of which 75 corresponded to genes known to condition bone metabolism. In particular OP bone had elevated levels of genes known to promote myelomonocytic/osteoclast precursor differentiation and osteoclast function

(*TREM2*, *ANXA2*, *SCARB2*, *CCL2/3*, *CD14*, *ST14*, *CCR1*, *ADAM9*, and *PTK9)* and a few downregulated genes that encode inhibitors of monocyte numbers/activity (*MNDA*, *PSTP1P1*, and *ADORA2A*). These transcriptional changes are consistent with the physiological events during bone metabolism of OP patients characterized by an increase in: (1) the number of osteoclast precursors and mature osteoclasts; (2) osteoclast activity; and (3) bone resorption.[33] A second group of upregulated genes normally function to inhibit osteoblastogenesis (*CD14*, *ANXA2*, *ST14*, *SGK1*, *CTSB*, *FST*, *LGALS1*, *SPP1*, *SPARC*), consistent with decreased bone formation in OP. A third group of differentially expressed genes is involved in: (1) regulating adipogenesis, such as genes with proadipogenic functions (*SPARC*, *ANXA2*, *IL10*, *MARCO*, *CD14*, *PPP2cb*, *AEBP1*, *FST*, *CCL2*); (2) lipid metabolism (*SCARB2*, *MARCO*, *ADFP*, *FABP5*, *CD14*, *PPP2cb*, *PLA2G7*, *CAV2*, *MGLL*), and (3) glucose metabolism/glucose transport/insulin signaling/IGF1 signaling (*PEA15*, *ANXA2*, *SGK1*, *IGFBP7*, *PPP2CB*, *HSD11B1*, *CCL2*, *DOK4*). These findings were all consistent with the frequent occurrence of insulin resistance, type 2 diabetes, and obesity in OP.[33]

The expression of several genes previously identified as contributors to OP pathogenesis, such as *TREM2*, *IL10*, *CD14*, *COL4A1*, *FST*, *SPARC*, *SHIP1*, and *SPP1*, were strongly differentiated by OP bone.[33] In addition, *IL1B*, *CALCRL*, *IL6*, *PLOD2*, and *RANK* were also significantly altered, but to a slightly lesser extent. Ten genes previously linked to OP through gene expression and animal models were not identified as genes differentially expressed by microarray analysis in this study (*RUNX2*, *LEP*, *VDR*, *ESR1*, *CALCA*, *CALCR*, *PTH*, *PTHR1*, *IGF1*, *LRP5*).[33] Even though these candidate genes were not differentially expressed, many genes were differentially expressed that are known to interact with and/or modify some of these well characterized genes and pathways. For instance, differentially expressed *SHOX2*, *PDLIM4*, *HDAC4*, and *TAZ* have all been shown to interact with *RUNX2* and regulate its activity, suggesting that the *RUNX2* transcriptional regulatory balance could be compromised in OP via these factors.[33] These findings suggest the presence of a very complex interplay between the osteogenic, adipogenic, and haematopoietic cell populations of the bone microenvironment that might account for these differences among experiments.

A follow up study performed by Reppe et al.[34] examined a larger population of OP patients, to reduce the possible variation errors that may have occurred with the small sample size studied by Hopwood et al.[33,34] Reppe et al.[34] compared *trans*-iliac bone biopsies from 84 postmenopausal women with BMD variations [37 with OP, 11 with "osteopenia," and 36 with normal or high BMD scores as determined by Dual-Energy X-ray Absorptiometry (DXA)], and correlated gene expression with BMD in each woman patient. They found ~1600 genes among three sites (L1-L4, total hip, and femoral neck) to be differentially expressed in the OP patients. Of these, 142 differentially expressed genes were found to be strongly correlated with BMD, 4 of which showed strong inverse correlation to BMD (*ACSL3*, *NIPS-NAP3B*, *ABCA8*, and *DLEU2*), while 4 showed strong positive correlations with BMD (*DKK1*, *SOST*, *C10RF61*, and a probe for the human 28S ribosomal RNA gene).[34]

Other interesting upregulated genes included *SEC61B*, thought to interact with *RAMP4* (regulator of osteoclasts), and *WIF1*, a WNT inhibitory factor similar to *SOST* in its regulatory role in bone differentiation.[96,97] Only 1 of these 142 differentially expressed genes overlapped with the Hopwood et al.[33] findings, *PDK4* (involved in glucose metabolism). Possible reasons for this discrepancy include the difference in sample size (10 vs. 37, respectively) and the differences in the comparison groups. Hopwood et al.[33] compared OP women to osteoarthritic or autopsy material, while Reppe et al.[34] compared patients with varying BMD and correlated that information with microarray chips that contained 3 times as many probes. In addition, the ethnic background of the patients should also be taken into account; Hopwood and coworkers' cohorts were Australian while Reppe and coworkers' biopsies were Norwegians.[33,34]

In a different study, Jemtland et al.[35] examined gene expression profiles of 84 postmenopausal women with distinct BMD values (27 OP T-score < 2.5; 18 with intermediate BMD values, and 39 healthy controls).[35] Of the 609 differently expressed genes, 439 were significantly upregulated and 170 were significantly downregulated in the OP patients; bioinformatics analyses defined 302 as disease-related, 47 as age-related, and 40 as bone mass index-related transcriptional changes.[35] Of the genes associated with OP, 256 were unique for being OP-related without being influenced by either age or bone mass index, indicating these particular genes as OP-specific candidate genes. Correlation analyses between these 256 candidate genes and BMD at 3 skeletal sites (hip, lumbar spine, and femoral neck) found that all 256 genes significantly correlated with total-hip BMD, 93% correlated with lumbar spine BMD, and 87% correlated with femoral neck BMD. This confirmed that the majority of OP genes are correlated with BMD. Some interesting genes found to be positively correlated with BMD in all three BMD phenotypes included *SOST* and *DKK1*, (both inhibitors of the canonical WNT signaling pathway; also shown to be upregulated in Reppe et al.[34]), *PGGF1* (which maintains the transcriptionally repressive state of *BCL6* and *CDKN1A*, both of which are involved in cell cycling and found to be differentially expressed in the Trost et al.[32] publication), and *PPME1*

[a protein phosphatase known to demethylate *PP2CB* (found by Hopwood et al.[33] to be differently expressed in OP patients[33])]. In addition, both *BCL2* and *SOX4* (thought to mediate *PTH* and *PTHRP* related bone development) were positively correlated with BMD at the hip and femoral neck but not at the lumbar spine. Finally, *KTN1*, *PTP4A1*, *RTN4*, *SLC16A1*, and *COPS4* were among the top 20 genes negatively correlated with BMD in all three phenotypes and all of which were also significantly decreased in Reppe et al.,[34] indicating their importance in OP.

1.5 Profiling Noncoding RNA Expression in Bone

MicroRNAs (miRNAs) are short (~20–25 nt long), single-stranded noncoding RNAs that play a major role in posttranscriptional gene regulation. Recent studies have shown that miRNAs play a role in regulating skeletal development and bone metabolism. For example, cartilage cell-specific knockdown of Dicer, an essential component of miRNA biogenesis, in mice, resulted in severe skeletal defects during development.[98] Inhibition of miR-92a enhanced fracture healing via promoting angiogenesis.[99] Transgenic mice overexpressing miR-206 in osteoblasts developed a LBM phenotype due to impaired osteoblast differentiation.[100]

Several in vitro[22,101–108] and in vivo gene expression profiling studies[36–38] have been conducted to identify miRNAs involved in the regulation of bone metabolism and bone diseases. In a recent study, using miRNA PCR Arrays, Seeliger et al. identified 11 miRNAs (miR-21-5p, miR-23-3p, miR-24-3p, miR-25-3p, miR-27a-3p, miR-100-5p, miR-122a-5p, miR-124-3p, miR-125b-5p, miR-148a-3p, miR-223-3p) significantly upregulated in the serum of osteoporotic patients compared with non-osteoporotic patients.[38] Five of these miRNAs (miR-21, miR-23a, miR-24, miR-100, miR-125b) were also found to be upregulated in the bone of osteoporotic patients. In another study, An et al.[36] investigated the changes in miRNA profiles in OVX mice and identified 9 miRNAs significantly differentially regulated between OVX and sham-operated mice. miRNAs miR-127, miR-133a, miR-133a*, miR-133b, miR-136, miR-206, miR-378, and miR-378* were identified as upregulated in OVX compared to sham-operated controls mice while, miR-204 was downregulated. Subsequently, they studied the role two of these miRNAs (miR-127; miR-136) have in osteoblast and osteoclast differentiation, as well as in osteocytes. Their results suggest that both miR-127 and miR-136 may contribute to bone loss by suppressing osteoblast differentiation and osteocyte survival, while promoting osteoclast differentiation.

Waki et al.[37] studied the differential regulation of miRNAs in healing femoral shaft fractures compared to unhealing fractures, in rats. This study identified 317 miRNAs with high expression in standard healing fractures compared to unhealing fractures. Waki et al. further filtered the genes based on fold upregulation and high expression and identified eight miRNAs (miR-140-3p, miR-140-5p, miR-181a-5p, miR-181d-5p, miR-208b-3p, miR-451a, miR-743b-5p, miR-879-3p) highly expressed in healing fractures compared to unhealing fractures. Five of these miRNAs (miR-140-3p, miR-140-5p, miR-181a-5p, miR-181d-5p, and miR-451a) were conserved between human and rat. Subsequently, they validated the expression of these miRNAs using reverse-transcription PCR and confirmed that all five miRNAs are significantly higher in standard healing fractures compared with unhealing fractures. Four of these miRNAs (miR-140-5p, miR-181a-5p, miR-181d-5p, miR-451a) have been reported to be involved in the regulation of skeletal development. Three miRNAs (miR-140-3p, miR-181a-5p, miR-451a) have been shown to regulate inflammatory responses.

Long noncoding RNAs (lncRNAs) belong to a class of noncoding RNAs, with transcript length ranging from 200 nt to ~100 kb. LncRNAs play a major role in regulating gene expression during development and disease. In recent years, a number of studies have been performed to identify novel lncRNAs in different cell types and diseases and to understand their function. However, very little work has been done in exploring the role of lncRNAs in regulating skeletal development and diseases, and much of this has been done in vitro. In a recent study, Wang et al.[39] profiled lncRNA expression during osteogenic differentiation of human bone marrow MSCs. Compared to day 0 (undifferentiated MSC), 1206 lncRNAs were significantly differentially expressed (687 upregulated and 519 downregulated) at day 14. Five of these lncRNAs (H19, uc022axw.1, XLOC_008374, LINC00707 and ENST00000436742.1) were further validated using qRT-PCR. A recent study by Huang et al.[109] showed that H19 promotes osteogenesis via TGF-β1/Smad3/HDAC signaling pathway, highlighting its role in the osteogenic differentiation process. Zuo et al.[40] profiled lncRNA expression during BMP2-induced osteoblast differentiation of C3H10T1/2 cells. At 1 day posttreatment, they identified 886 upregulated and 825 downregulated lncRNAs in the BMP2 treated group. 595 upregulated and 548 downregulated lncRNAs were identified at day 4 posttreatment. 59 upregulated and 57 downregulated lncRNAs were common to both time points. These studies have identified several miRNAs and lncRNAs with potential role in bone metabolism, OP and fracture healing; however, functions of majority of these noncoding RNAs remain unclear. Further in vivo studies are required to define the precise role of each of these miRNAs and lncRNAs in bone development and diseases.

2 CONCLUSIONS

In this report, we highlighted a few key publications spanning the last decade that cover key topics in bone biology that have greatly benefited from gene expression profiling. The summary of the genes discussed is also presented in Table 5.1. There are many more in the literature, and by no means have we exhausted the published data. The microarray and RNAseq experiments we have outlined earlier clearly emphasize that it is no easy task to explain, based on genomic profiling how bones respond to loading or maintain their integrity. Skeletal tissue is highly complex both molecularly and physiologically. And while variations in transcript levels for a small combination of genes have the potential to lead to altered bone metabolism, and may ultimately result in bone loss phenotypes, these gene expression experiments clearly identify larger cohorts of transcripts, some of which may not be the obvious candidates for the job, according to their previously described functions. There are limitations on how we can design and generate large datasets based on in vivo experiments. As of now, we can only obtain static glimpses of the bone microenvironment, quantifying the transcriptome incrementally as a function of treatment or disease. As we learn how to measure these events dynamically in humans and animal models, we will be able to determine how the disease emerges or progresses. These genome-wide expression studies undoubtedly will help identify multiple new candidate genes in an unbiased approach but, as for now, these putative candidates still require significant validation through traditional experimental approaches, which will continue to be the bottleneck of scientific discoveries. While the field of bone biology is still in its infancy exploring the available genome-wide technologies, it can greatly benefit from the existing body of work and can move forward with unique and creative approaches that will ultimately lead to well designed and successful gene expression experiments.

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CHAPTER

6

Functional Genomics

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1 WHAT IS FUNCTIONAL GENOMICS?

The publication of the first draft sequence of the human genome marked a milestone in our global understanding of human biology.[1,2] It provided a comprehensive catalog of the 3 billion nucleotide sequence. One of the mysteries was that the roughly 20,000 protein-coding genes occupy only a small fraction (about 1%–2%) of the whole genome. What are the roles of the remaining 98%–99% of the genome, which were once regarded as "junk"? Functional genomics can be defined as studies designed to assign function to this wealth of genomic information. Large international efforts, such as ENCODE and the Epigenomics Roadmap have since revealed hundreds of thousands of regulatory elements throughout the genome that reflect the complexity of gene regulation, and these elements are often located in these intergenic regions. Functional domains in the genome include protein-coding genes with their promoters, exons, and introns, in addition to enhancers, insulators, and imprinting control regions. Many of these regulatory elements are enhancers, which interact with promoters of genes and regulate both spatial and temporary patterns of gene expression.

Another layer of complexity was added with the discovery of the three-dimensional organization of the genome. The genome is organized into so-called topological domains, which are megabase-sized local chromatin interaction domains separated from each other by the insulator protein CTCF.[3] These domains, which are highly conserved between mice and men, separate different transcriptional control processes. Surprisingly, these domains are relatively similar between different cells. Disruption of the barriers between topological domains has been shown to be a mechanism in certain malignancies.[4]

Cell-specific maps of the regulatory elements in our genome can be layered onto the DNA sequence information, bringing life to the "simple" DNA map of sequences and providing a look into the inner workings of a given cell. However, novel approaches are needed to integrate and better understand the function of genomic and epigenomic information. In addition to a better understanding of the physiology of the bone cells, these advances are already providing help in our search for the basis of skeletal diseases and bone cancer.

This chapter addresses methods and recent advances in functional genomics. We describe the identification of functional elements throughout the genome and provide examples with relevance to the field of bone and mineral metabolism. We then highlight examples of methods to assess their cellular function. As this chapter can only mention a small part of the rich published literature, the reader is referred to other chapters in this book and recent reviews.

2 ANNOTATING THE GENOME—AN EMERGING PICTURE

2.1 Encode and Roadmap: An Overview

ENCODE, the Encyclopedia of DNA Elements, is a massive NIH-sponsored public research project launched in 2003 as a follow up of the Human Genome Project. Its goal is to better understand the vast majority of noncoding genomic DNA and to comprehensively catalog functional elements in the human genome. The primary methods used were DNase-seq, RNA-seq, ChIP-seq, and assays for DNA methylation. After an initial pilot phase using 1% of the human genome, the consortium applied their methods to the entire genome in the production phase. The initial results were published in 2012 comprising 30 papers in *Nature*, *Genome Research*, and *Genome Biology*.[5] Taken the results from all 147 investigated cell types, more than 80% of the genome is involved in at least one chromatin or RNA-associated event. More than 70,000 promoters and 400,000 enhancers were assigned.

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Intriguingly, single-nucleotide polymorphisms found in genome-wide association studies (GWAS) to be associated with complex traits are enriched in noncoding regions that contain ENCODE-annotated functional elements. This has created opportunities to understand GWAS findings and has already resulted in some spectacular findings.[6] ENCODE also determined that much of our genome is transcribed into noncoding RNA molecules, and some of them are now known to belong to the large group of noncoding RNA of regulatory elements.[7] The ENCODE findings also contribute to our understanding of the evolution of regulatory elements. Contrary to previous assumptions, many enhancers are located in regions that are not the most evolutionarily conserved ones, pointing to their relatively recent development. The current phase of the ENCODE project is providing an even deeper characterization of our genome.

The complementary efforts of the NIH Roadmap Epigenomics Mapping Consortium generated high-quality, genome-wide epigenomic data (histone modification, DNase hypersensitivity sites, DNA methylation, and RNA expression) across 100 different human cell types and human tissues.[8] The current dataset of ENCODE and Roadmap contain limited data from bone cells, namely only partial datasets for primary human osteoblasts (ENCODE uses the name E129). They include genome-wide methylation profiles, histone modifications (such as H2A.Z, H3K27ac, and H3K4me1), and CTCF insulator sites (http://www.encode-roadmap.org). ENCODE and Roadmap data can be easily visualized or downloaded using genome browsers, such as the UCSC genome browser.

2.2 DNase-seq

DNase-seq is a powerful technique that produces a global view of open chromatin, and allows for the prediction of specific transcription factor (TF) binding sites. DNase-seq is based on the well-established observation that within the genome, active DNA elements (promoters and enhancers) exist in an "open" (i.e., nucleosome-free) chromatin configuration making the binding of TFs to DNA possible (Fig. 6.1).[9]

In contrast to "closed" chromatin, these "open" sites can be digested with DNase I; the location of these DNase hypersensitivity sites (DHS) can then be mapped to the genome, revealing active regulatory elements.[10] Recently, next generation sequencing of the DHS was shown to allow for a surprising and unexpected resolution of TF binding sites down to the base-pair level. This resolution is based on the fact that within "open" DHS (median size 300 bp), individual base pairs bound by their specific TF are protected from digestion by DNase I, and, as a result, very specific "footprints" are left behind.[11–14] Putative binding sites for specific TFs are identified among these footprints by computational means using the consensus DNA-binding sequences of the TF of interest.[15] Data from DNase-seq has been demonstrated to have striking concordance with ChIP-seq and crystallography data, for instance across 41 diverse cell and tissue types by ENCODE.[16]

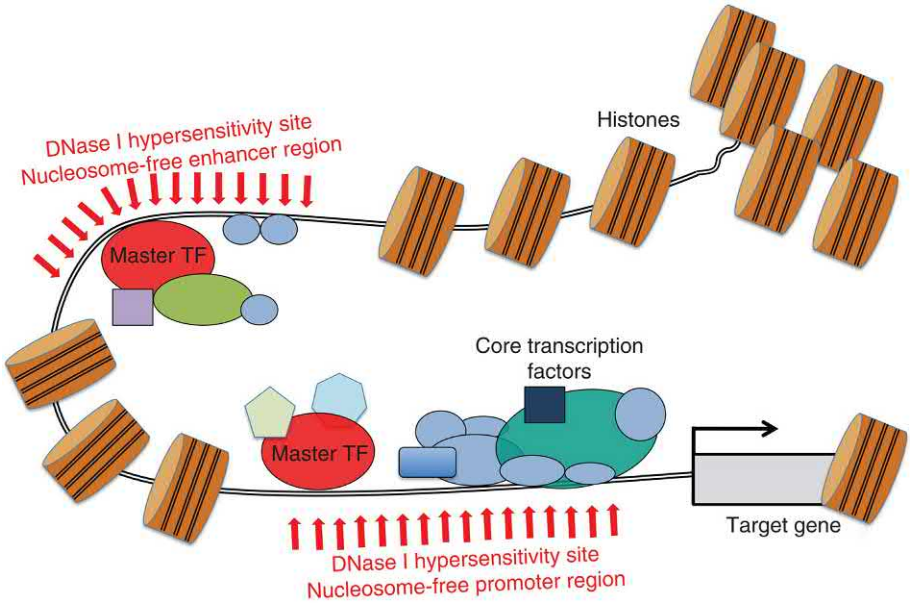

FIGURE 6.1 **Schematic depiction of the general genomic organization with gene body, proximal promoter, and distal element (enhancer).** DNA is wrapped around histone proteins. Inactive regions of the genome are tightly bound by histones and condensed, and inaccessible to DNase I digestion. In the open configuration, DNA is exposed and susceptible to digestion with DNase (so-called DNase-I hypersensitivity sites). DNase hypersensitivity sites (DHS) are typically related to functional activity with various proteins and transcription factors (TFs) binding to these sites.

2.3 ChIP-seq and Chromatin Profiling

All sites of chromatin modifications or TFs binding can be mapped using chromatin immunoprecipitation combined with massively parallel sequencing (ChIP-seq). This powerful method makes it possible to understand differences between both tissues and cells, and between different cell differentiation states. For example, the changing epigenomic landscape can be addressed when osteoblasts differentiate into osteocytes. By determining the chromatin marks associated with active promoters (H3K4me3 and H3K9ac), active enhancers (H3K4me1 and H3K27ac), or active repressors (H3K27me3 and H3K9me9), a deep view into the changing usage of promoters and enhancers down to several base-pair levels can be obtained. This example is further discussed later.

In brief, to carry out ChIP-seq, cultured cells, or minced fresh or frozen tissue, are treated with formaldehyde, which crosslink TFs that are bound to their cognate DNA. Nuclear extracts are prepared, and genomic DNA is sheared using sonication. The resulting DNA–protein complexes are immunoprecipitated using a histone mark or TF-specific antibody. The immunoprecipitated chromatin is collected and crosslinks are reversed. Purified DNA is sequenced using next generation sequencing. DNA that was bound by the TF of interest is enriched in the sample and identified through direct sequencing. A positive control antibody and a negative control (e.g., preimmune serum) are used.

Several examples of epigenetic profiling of skeletal cells are given. These publications give an insight into the kind of data that can be generated using genome-wide epigenetic profiling.

2.3.1 SP7/Osterix is Restricted to Bone-Forming Vertebrates Where it Acts as a Dlx Cofactor in Osteoblast Specification

An example of the power of chromatin profiling combined with ChIP-seq data is the recent comprehensive analysis of osterix targets in osteoblasts.[17] Osterix (SP7), together with RUNX2, is one of the key TFs in the development of bone cells.[18] During skeletal development, mesenchymal progenitors develop into RUNX2-positive preosteoblasts, then become RUNX2/osterix-double positive osteoblast precursors and later mature osteoblasts. The critical importance of osterix for the development of precursors into osteoblasts was demonstrated by the failure of osterix-null mice to develop mature osteoblasts.[19] Therefore, a better understanding of the molecular targets of osterix is fundamental for our understanding of osteoblast development.

Hojo et al. sought to identify targets of osterix, using primary osteoblasts from mice as opposed to a cell line to more faithfully represent the in vivo condition. To facilitate chromatin immunoprecipitation, they first generated a knocking mouse to add two tags (a biotinylated sequence and a 3xFLAG tag) to osterix expressed from the endogenous locus. Antibodies against the FLAG tag and streptavidin for biotinylated proteins are well suited for immunoprecipitation.

The analysis revealed key insights into the role of osterix. Genome wide, the investigators identified 2,112 osterix-binding peaks. Based on conservation scores, osteoblast expression of the nearest gene, and strength of the ChIP-seq peaks, 194 putative enhancer regions were identified. For a subset of them, enhancer activity was confirmed in cell-based luciferase reporter assays, and bone-specific expression confirmed using transgenic reporter mice. An example of the power of this approach to identify targets of osterix was provided by focusing on osteoblast-expressed genes with strong association with osterix, which included Notch2, FGFR2, COL1a2, Gli2, and Kremmen1. Notch2, which is expressed in all skeletal cell types, and of known importance for osteoblast differentiation, contains an osterix ChIP peak in intron 2 located in a conserved block of DNA. An enhancer-driven reporter containing DNA from this intronic region demonstrated higher activity in preosteoblastic MC3T3E1 cells compared with 3T3 cells. The activity of this enhancer increased several folds during osteogenic induction and was suppressed by osterix knockdown, consistent with an osteoblast-specific regulation through the regulatory region in intron 2 of Notch2 (Fig. 6.2).

Surprisingly, most osterix peaks did not map to a consensus GC box that is used by other members of the Sp family of TFs to bind DNA. However, in EMSA assays, osterix does not consistently bind to the AT-rich motif it was observed to associate with by ChIP-seq. This paradox was supported by evidence that suggests that osterix binds most targets indirectly by binding to homeodomain-containing TFs of the Dlx family, which then directly bind to DNA target

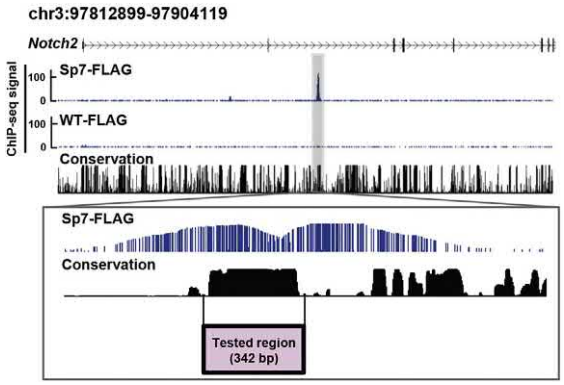

FIGURE 6.2 Osterix ChIP-peak identified in intron 2 of Notch2 in a conserved region. A 342-bp piece was tested in an enhancer–reporter assay, which showed strong correlation to osteogenic induction.[17]

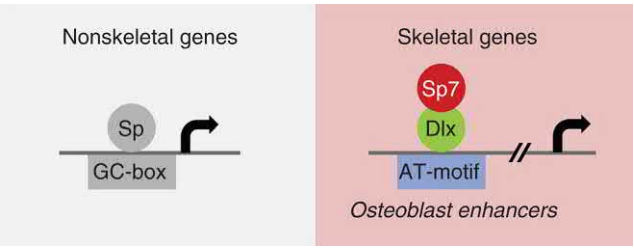

FIGURE 6.3 Model of Sp7 (osterix) binding to osteoblast enhancers of skeletal genes, as opposed to the typical mode of action of other members of the Sp family of TFs for nonskeletal genes.[17]

sequences. Furthermore, the identification of an N-terminal transcriptional activation domain in osterix led the authors to predict that osterix acts as a transcriptional coactivator for Dlx in osteoblast development. Paralogs to osterix were identified in all bone-forming vertebrates. The emergence of osterix (Sp7) within the Sp family, and its ability to enhance the evolutionary older transcriptional activity of Dlx factors might therefore have been a key factor in the development of bone-forming cells during evolution. This study provides an unprecedented insight into the osteoblast regulatory program, and novel mechanistic understanding of how osterix binds its targets (Fig. 6.3).

2.3.2 *Distinct Transcriptional Programs Underlie Sox9 Regulation of the Mammalian Chondrocyte*

Sox9 is an essential TF for chondrocyte specification and differentiation. Sox9 is expressed in cells of mesenchymal condensation and is essential for their initial differentiation into chondrocytes and osteoblasts.[20] After the initial differentiation, Sox9 is exclusively expressed in chondrocytes. In the growth plate, its expression is high in proliferating chondrocytes, but Sox9 expression is lost as chondrocytes differentiate into hypertrophic chondrocytes. The genome-wide identification of Sox9 targets has been an important step in our understanding of this key skeletal TF.[21] Using an in vivo approach, investigators subjected mouse rib chondrocytes to Sox9 ChIP-seq experiments. 27,656 peaks were identified genome wide and further analysis revealed two distinct types of Sox9 binding in the chondrocytes' genome. There were two classes of osterix target sites. About 25% of peaks were found around the transcription start site of genes, were associated with key components of the transcriptional complex, and included nonskeleton-specific genes, such as house-keeping genes. These so-called class I peaks showed relatively weak Sox9 binding, and importantly, did not show enrichment for Sox9 DNA binding motifs. The remaining peaks form class II targets, which showed typical enhancer signatures, as they were enriched in the histone marks (H3K27ac and H3K4me2). In contrast to class I peaks, Sox9 binding

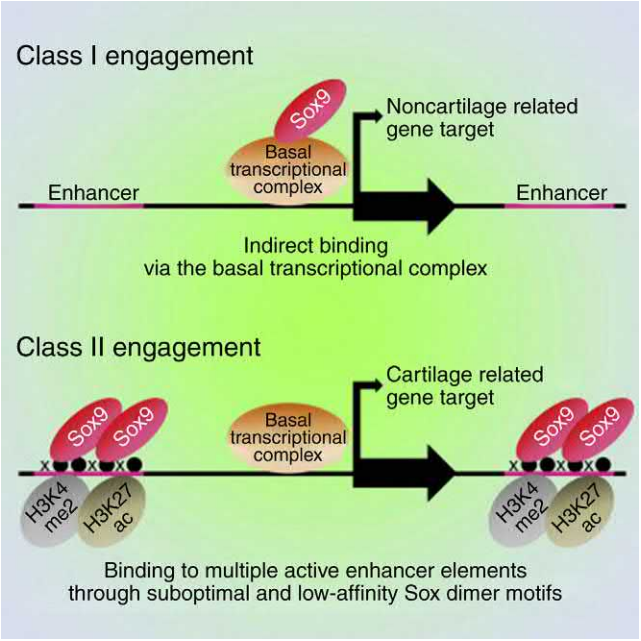

FIGURE 6.4 ChIP-seq using mouse chondrocytes revealed two different classes of Sox9 engagement: class I sites are located at or near the transcription start site (TSS) of house-keeping genes and part of the basal transcriptional complex; class II sites are enhancers of cartilage-specific genes.[21]

motifs were highly enriched in so-called class II peaks, and the associated genes were enriched in cartilage-related genes (Fig. 6.4).

In addition, 502 superenhancers around 422 genes were identified. These are special enhancers that lead to high levels of expression of their associated genes, and are often found near genes that code for cell-type-specific master TFs. Therefore, genome-wide mapping of Sox9 binding sites further clarified the mechanism through which it controls chondrocyte development.

2.3.3 *The Osteoblast-to-Osteocyte Transition: Epigenetic Changes and Response to the Vitamin D3 Hormone*

Osteocytes are derived from osteoblasts and possess unique characteristics. They are by far the most abundant cell type in the adult bone, and while they are embedded in mineral matrix, they maintain multiple connections to other cells in the bone and orchestrate bone remodeling. They produce factors, such as sclerostin and FGF23, play a role in bone resorption, and function as mechanosensors.[22]

A fundamental question in the understanding of osteocytes is the characterization of the changes that cells undergo during the transition from osteoblasts to osteocytes. Using the conditionally immortalized osteocyte cell line IDG-SW3, which has osteoblast-like characteristics at 33°C and develops into osteocytes at

37°C in osteogenic media, investigators profiled these cells in an attempt to obtain insights into the transition to osteocytes. Extensive ChIP-seq analysis of key histone marks across the genome at day 3 and day 35 of differentiation from osteoblasts into osteocytes were performed and compared with global changes of the transcriptome. Most changes of RNA abundance were reflected by the expected quantitative changes of histone modifications for gene elongation and gene enhancer activities. However, a limited number of histone modifications were either removed or added, for example, in the gene locus for PTH1 receptor and osterix (Sp7).[23]

2.3.4 The Binding of Runx2 During Osteoblastogenesis

Runx2 is a master regulator of osteoblastogenesis and mice missing Runx2 have no osteoblasts, and abnormalities in chondrocytes.[20,24] The genome-wide identification of target genes would therefore provide an important step for a better understanding of its transcriptional program. Using ChIP-seq, investigators recently profiled Runx2 binding using MC3T3-E1 preosteoblasts during proliferating, matrix deposition, and mineralization.[25] Binding of Runx2 to promoter regions, and to presumed enhancers were identified, with significant changes during differentiation. Runx2 binding was found in genes known to be regulated by Runx2, and also in genes of broad general cellular processes. These investigations led to the identification of new Runx2-regulated genes and extend our understanding of Runx2-regulated gene network.

2.3.5 Chromatin Profiling of Parathyroid Glands

The methods in functional genomics described earlier can be applied to other cells and tissues that play a role in skeletal and mineral homeostasis. For example, there is a lack of knowledge of the specific transcriptional program that governs parathyroid cells. It is not known, what epigenetic changes are characteristic of adenoma of primary hyperparathyroidism, and a comprehensive understanding of target genes of the parathyroid-specific master TF GCM2 is missing. We have therefore undertaken DNase-seq and chromatin profiling of human parathyroid glands, as well as ChiP-seq experiments using antibodies against GCM2. Together with transcriptome data these approaches will annotate parathyroid-specific active elements in the genome as a first step toward further insights into the transcriptional regulation of parathyroids cells (Fig. 6.5).

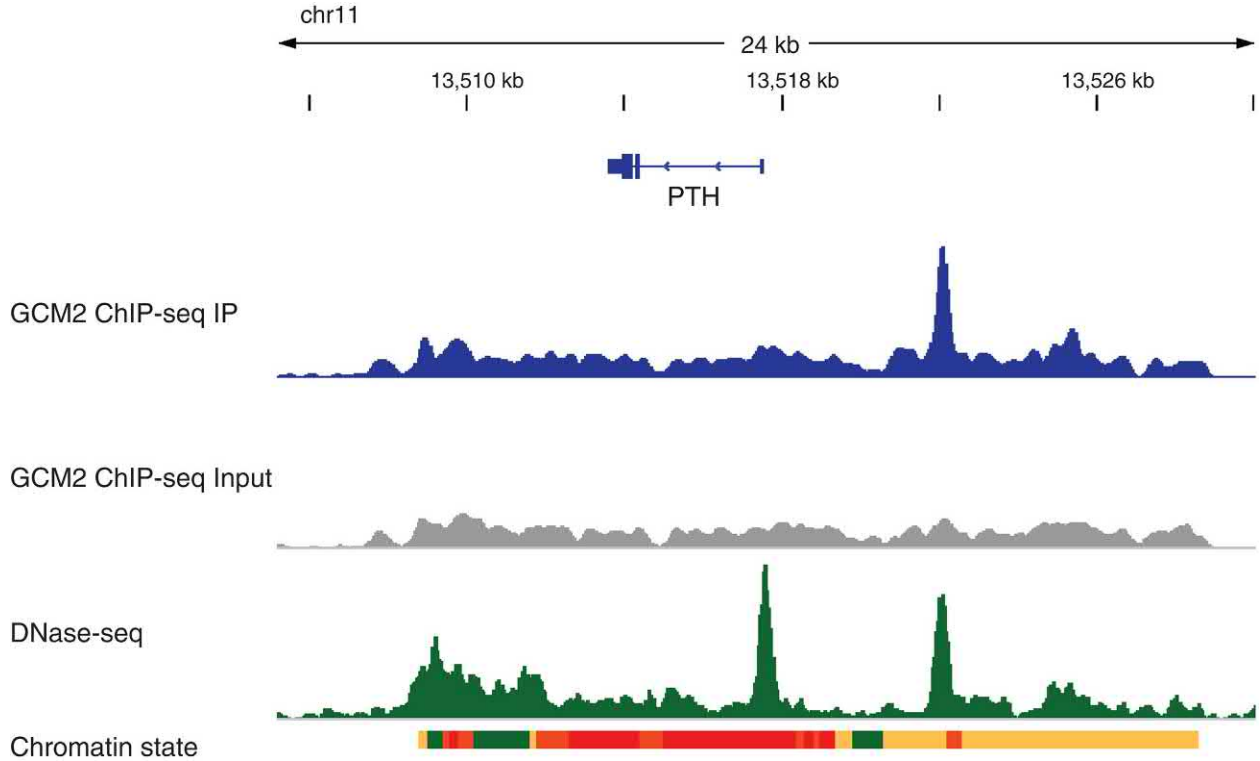

FIGURE 6.5 **DNase-seq data, GCM2 ChIP-seq, and chromatin states obtained from human parathyroid tissue (unpublished data).** Browser view showing broad peaks of DNase hypersensitivity at the proximal PTH promoter, over the gene body, as well as 5 kb upstream of the TSS, and GCM2 engagement 5 kb upstream of the TSS.

3 FROM ANNOTATED SEQUENCES TO FUNCTION

Fifteen years ago, a draft of the human genome was first reported.[1] Since then, biomedical research has been revolutionized by rapidly increasing knowledge regarding the genetic basis of disease and variation across individuals.[26] In contrast, our understanding of the functional consequences of such variation has lagged far behind the accelerated rate of gene and locus discovery afforded by the genomic revolution.[27] Broadly speaking, functional genomics refers to studies designed to assign function to this wealth of genomic information.

Well-powered GWAS have been performed to understand how common genetic variants contribute to many traits pertinent to mineral metabolism[28,29] and skeletal biology.[30,31] While these studies identified variants in genes previously known to regulate these traits, functional characterization of novel genes identified has been lacking. As such, a major unmet need is to comprehensively use the clues provided from human genetics to obtain a better understanding of the molecular circuitry controlling the cells crucial for mineral metabolism and skeletal homeostasis. Here, we will review recent technical advances in the area of functional genomics that will allow comprehensive characterization of such variants.

As discussed earlier, the ENCODE project[32] was launched in 2003, and sought to identify all functional elements in the human genome. This included a mammoth effort to map epigenomic modifications at the genomic level. Currently, information regarding nucleosome accessibility, histone tail modifications, TF binding sites, long-range intra- and interchromosomal interactions, and expression (transcriptomic) profiling is available across a range of human cell and tissue types.

Facilitated by advances in next generation sequencing technology, these efforts have rapidly led to the development of annotated maps that can be layered on top of genomic sequences. This work has led to an explosion in ideas, fueled by bioinformatics, about the functions of the noncoding genome. The majority of GWAS-identified trait and disease variants do not affect protein-coding DNA sequences[33]; therefore, a likely possibility is that noncoding variants linked to disease affect the function of regulatory elements present in the noncoding genome. Importantly, cell and tissue types crucial for mineral metabolism and skeletal development are under represented in current ENCODE databases.

In addition, information provided by ENCODE only provides a snapshot of the epigenomic state of the genome in whatever cell or tissue type is analyzed at a given developmental stage. This information generates hypotheses about the function of noncoding sequences and an embedded disease-associated variant, but functional data remains needed to truly understand how noncoding nucleotide changes affect cellular phenotypes. Here, we will review current efforts outside the field of skeletal biology to approach this daunting problem, and discuss how these conceptual approaches might be applied to bone biology and mineral metabolism research.

As the majority of GWAS variants associated with disease risk and trait variation lie outside of protein-coding DNA sequences, novel techniques are needed to better understand how sequence variation affects cellular and organismal phenotypes. One approach to this challenge has been to crossreference ENCODE annotation to disease-associated noncoding variants. However, several caveats apply to this approach.

First, the cell type(s) responsible for the phenotypic effects of a given variant are not necessarily known. Furthermore, epigenomic datasets from ENCODE are incomplete in that many cell types of particular importance in skeletal biology and mineral metabolism are not represented. Therefore, optimal annotation in the correct cellular context may be absent. For example, a distinct possibility is that common variants associated with differences in height[30] modulate the pathways underlying chondrocyte hypertrophy in the growth plate.[20] As growth plate chondrocytes have not been extensively profiled in ENCODE, cell type specific epigenomic marks linked to common sequence variation may be missed.

Second, ENCODE annotation can provide information about the broader regulatory state of a given region of noncoding DNA. However, the function of individual nucleotides within such regions cannot be determined. Therefore, higher-resolution maps of the function of the noncoding genome are required.

Third, epigenomic mapping of noncoding sequences provides hypotheses about the potential function of these sequences, many of which have borne out to be correct when tested in tradition, "low-throughput" experimental systems.[34–36] However, these predictions have not been extensively tested; therefore, at the present time functional data are needed to fully understand how a sequence variant might affect the function of a noncoding regulatory DNA element.

Here, we will summarize two recently described approaches that begin to address this important challenge.

3.1 In Situ Saturating Mutagenesis With CRISPR/Cas9

As outlined earlier, sequence variants associated with disease risk and phenotypic variation are enriched in noncoding enhancers. However, fine resolution functional understanding of sequence elements across lengthy stretches of enhancer DNA has been difficult to achieve. The advent of genome editing with CRISPR/Cas9[37] has

revolutionized our ability to experimentally interrogate the function of the noncoding genome.[38]

Bauer et al. recently reported the use of in situ saturating mutagenesis to dissect disease-associated enhancer function.[39] Hemoglobinopathies, such as sickle cell anemia are due to mutations in the beta-globin gene. Persistent expression of the fetal hemoglobin (HbF) variant is known to be protective for sickle cell disease. Genetic studies have implicated a crucial role for the transcriptional repressor BCL11A as a regulator of HbF expression.[40] BCL11A itself is transcriptionally regulated by an erythroid-specific enhancer, which is subject to noncoding genetic variation in humans.[41]

However, it had been difficult to precisely map the functions of multiple potential BCL11A enhancers with traditional methods. For this reason, a saturating mutagenesis screen was performed. To do this, Canver et al.[39] designed *all* possible sgRNAs targeting the putative human and murine BCL11A enhancers and introduced these sgRNAs along with active Cas9 into erythroid lineage cells via lentiviral-mediated transduction. The typical outcome following Cas9-mediated double stranded DNA breaks is repair via the imprecise nonhomologous end joining pathway; therefore, a panel of insertions/deletions will be generated by this in situ mutagenesis approach. Phenotypic effects of these noncoding mutations were assessed based on HbF expression. Cells were then analyzed for HbF expression by flow cytometry, and cells HbF^{hi} cells were recovered by cell sorting. Genomic DNA isolated from these cells was purified, and sgRNA sequences present (due to lentiviral integration into the host cell genome) characterized by next generation sequencing and their abundance compared to the input population of sgRNAs tested. This approach allowed quantification of the effects of all 1973 sgRNAs on regulation of HbF expression.

Two interesting and unexpected results were noted. First, in each species a single subelement of BCL11A enhancer DNA was observed to have a dominant effect in regulating BCL11A (and therefore HbF) expression. In humans, ~4 kb of putative enhancer DNA was tested, and yet mutation of only ~350 bp of this sequence led to appreciable effects. Therefore, only a subset of DNase-hypersensitive enhancer sequence is "essential" for BCL11A expression as defined by this technique. Second, although both human and murine BCL11A share conserved intronic enhancer elements, the subelement deemed to be most important for BCL11A expression differed across species. Therefore, species-specific studies should be conducted when assessing effects of noncoding elements in the future, as apparent sequence conservation appears to mask underlying functional divergence.

This powerful approach of saturating mutagenesis via CRISPR/Cas9 can be broadly applied to dissect noncoding DNA function across genes and cell types. In bone, for example, a number of polymorphisms near the SOST gene (encoding the osteoporosis drug target sclerostin) have been linked to bone mineral density and fracture risk.[31] However, our understanding of the regulatory elements controlling SOST expression in murine and human cells remains incomplete.[42,43] Applying a similar in situ saturating mutagenesis screen to better understand the functional impact of elements controlling SOST would significantly improve our understanding of how this gene is regulated at the transcriptional level, and how human genetic variation linked to skeletal phenotypes intersects with the circuitry controlling SOST expression.

3.2 Multiplexed Reporter Assays (MPRA) to Assess Function of Expression-Modulating Variants

The in situ saturating mutagenesis approach outlined earlier powerfully assesses function of enhancers at the single-nucleotide resolution. However, this method currently focuses on a relatively small stretch of noncoding DNA (4–5 kb per species), and pays no attention to the impact of common genetic variants.

Recently, two groups have described an alternative and complementary method to assess functional effects of single nucleotide variants through parallel large-scale episomal reporter assays.[44,45] Here, large numbers of sequences encompassing variants linked to different phenotypic traits (red blood cell parameters, inflammatory bowel disease, BMI, coronary artery disease, among others) were cloned into barcoded reporter vectors. For each putative disease-associated variant, both common alleles are tested in parallel. Next, bulk populations of these reporter constructs were introduced into cells of interest, and reporter activity was determined by next generation sequencing. This workflow allows for systematic interrogation of whether disease- or trait-associated variants influence reporter activity.

This approach provides a vital framework for future studies to better understand the function of sequence variants linked to disease. Further study of all variants identified as functionally important from this approach is required. Merging multiplexed reporter assays (MPRA) data with ENCODE annotation and expression data appears to highlight variants of particular functional importance.[45] Overall, it is quite clear that MPRA represents a powerful addition to the current toolset of GWAS follow-up techniques. Again, one can imagine how this framework could be applied to efforts to better understand how variants associated with skeletal and mineral metabolism traits exert their functional effects. For example, a MPRA library could be generated to include all bone mineral density-associated variants, and

subsequently this bone-focused library could be profiled in osteoblastic and osteoclastic cells.

4 INTERROGATION OF CELLULAR FUNCTION: GENOME-WIDE GAIN- AND LOSS-OF-FUNCTION SCREENING IN MAMMALIAN CELLS

Without a doubt, a better understanding of how common human genetic variants linked to disease function is a major priority in the rapidly developing field of functional genomics. However, the magnitude of phenotypes caused by most GWAS "hits" is rather modest. Furthermore, common variants (and thus GWAS hits) are limited in effect size due to absence of evolutionary selection. Therefore, complementary approaches to interrogate cellular function at the genome-wide level are needed.

For decades, investigators in our field have employed powerful cell culture models to interrogate mechanisms of chondrocyte,[46] osteoblast,[47] osteoclast,[48] and osteocyte[49] functions in vitro in transformed cell lines. While all these models do have limitations, they have yielded crucial bona fide insights into cellular function. In addition, recent advances in induced pluripotent stem cell technologies have led to derivation of osteoblasts[50] and chondrocytes[51] from these sources. Therefore, a wide variety of options are now available to explore molecular mechanisms in bone cell function in vitro.

The vast majority of mechanistic studies pursued to date in such systems have involved detailed characterization of candidate genes of interest. Such studies are inherently limited by existing knowledge. Historically, genetic screens have been performed in model organisms, such as baker's yeast, worms, and fruit flies.[52–54] These unbiased approaches have yielded important new insights into a wide variety of biologic processes. More recently, multiple methodologies have been developed to perform functional genetic screening in cultured mammalian cells. Here, we will review genome-wide functional screening methodologies, including RNA interference (RNAi), ORFeomics, and CRISPR/Cas9, and discuss applications to probe mechanisms of bone cell function.

4.1 RNAi-Based Functional Genomics

RNAi refers to sequence-specific posttranscriptional gene silencing. This is initiated by a double-stranded RNA species homologous to the sequence of the silenced gene. Over 15 years ago, it was demonstrated that RNAi is mediated by sequence-specific 21-nucleotide siRNA duplexes, which could easily be delivered to mammalian cells.[55] Since then, RNAi has proved to be an invaluable tool for experimental loss-of-function studies in a wide variety of mammalian cell lines and primary cells. Given the refractory nature of some primary cells to transfect with RNAi duplexes, alternative delivery methods, such as lentiviral-mediated shRNA expression, have been developed.[56] In skeletal biology, lentiviral-mediated shRNA has been described for selected target gene silencing in a variety of cells, including primary calvarial osteoblasts.[57] Shortly after its introduction as a tool to disrupt target gene expression for focused candidate gene studies, large-scale RNAi libraries were developed to facilitate genome-wide functional screens.[56,58] In such studies, libraries of RNAi or shRNA constructs are generated, introduced into target cells, and then phenotypic consequences assessed.

While the majority of large-scale screens have been performed in cancer cells and neurons, to date two screens in bone cells have been reported, both in human bone marrow derived mesenchymal stromal cells capable of osteoblast differentiation.

Zhao and Ding[59] screened a synthetic siRNA library targeting 5000 human genes to identify repressors of osteoblast differentiation. Mesenchymal stromal cells were transfected with this library in high-throughput conditions, and cells were subsequently assayed for alkaline phosphatase (ALP) expression as an early marker of osteoblast differentiation. Importantly, cells were grown under conditions not designed to promote robust osteoblast differentiation. The authors reported 53 confirmed "hits" that gave rise to a significant increase in ALP expression. Twelve of these genes were selected for further studies in vitro. Interestingly, this approach identified GNAS as a gene that regulates osteoblast differentiation, a result corroborated by extensive in vivo human and mouse genetics.[60,61]

More recently, Zou et al.[62] reported a similar strategy in which the same human mesenchymal stromal cells were screened with a lentiviral-based shRNA library targeting ~1500 human kinases, phosphatases, and receptors. Importantly, this library contains four to five distinct shRNAs for each gene targeted, allowing for confirmation that multiple (at least two) independent shRNAs cause the same phenotype. While systematic results of all genes whose knockdown causes increased ALP was not reported, DCAMKL1 emerged as a robust "hit" from this screen. The authors went on to demonstrate that DCAMKL1 knockout mice show increased bone mass and osteoblast activity, as predicted from the shRNA screening results. Furthermore, DCAMKL1 controls activity of the TF RUNX2 in vitro, and a genetic interaction between DCAMKL1 and RUNX2 was demonstrated in vivo.

Comparing these two similar screens side by side is challenging. Zhao and Ding did not report DCAMKL1 as a "hit" from their RNAi screen, but it is not clear if DCAMKL1 was present in the library of RNAi duplexes

tested. Zou et al. did not comment on whether any of the 53 "hits" from the Zhao and Ding study were detected in their screen, and did not report a full list of genes whose knockdown increased ALP in their study. These shortcomings illustrate some of the challenges associated with RNAi/shRNA screening, systematic data analysis and sharing tools would greatly facilitate efforts to clarify the rigor and reproducibility of "hits" identified. To the great credit of Zou et al.,[62] the shRNA screen reported represented a starting point for detailed functional studies on a new gene, DCAMKL1, and its role in bone biology.

Recently, it has become clear that major caveats associated with RNAi screening exist. Many potential drug targets identified through this technology in academic laboratories have not been confirmed by independent studies in industrial laboratories.[63] In addition, an inherent weakness in RNAi-based screening is that the introduced small RNA enters the microRNA processing pathway, and therefore affects expression of many mRNAs that have a complementary "seed" (first 9 nucleotides) rather than the full 21 nucleotides of complementarity.[64] The pervasiveness of such "off-target" effects in the setting of RNAi screening is now well-appreciated.[65] These findings have led experts in the field to conclude that the "honeymoon" is over with respect to current RNAi screening technologies.[66] Certainly, relying on a single RNAi sequence as a surrogate for a gene's function may lead to erroneous conclusions. Proper interpretation of RNAi screening experiments therefore requires robust computational approaches, including assurance that multiple independent RNAi sequences targeting a single gene all leads to the same phenotype. Therefore, novel modalities are needed for robust functional genomic screening in mammalian cells.

4.2 The Orfeome: A Tool for Gain-of-Function Screening

While generally loss-of-function approaches are thought to be ideal to define gene function, complementary gain-of-function studies are also useful in certain instances. Large-scale libraries of cloned cDNAs for overexpression studies have been developed to facilitate such efforts.[67,68] Yang et al. have reported the largest collection of cloned open reading frames (ORFs) to date, that is, >16,000 cDNAs inserted into a versatile epitope-tagged lentiviral expression vector. This collection also benefits from a barcoded tag to easily facilitate identification of cDNAs leading to cellular phenotypes from genomic DNA of infected cells by next generation sequencing. Gain-of-function screens have been reported with this library, mainly focusing on identifying kinases that when overexpressed lead to resistance to chemotherapy effects in cancer cell lines.[69]

To date, no such "ORFeomic" screens have been performed relevant to skeletal biology. Incomplete knowledge exists regarding the regulatory mechanisms controlling expression of several bone-specific genes. Therefore, one could envision that a gain-of-function screen designed to identify new genes sufficient to induce high expression of FGF23, for example, could lead to important and novel insights into the mechanisms controlling phosphate homeostasis by bone cells.

4.3 Genetic Screens Using CRISPR/Cas9: The Next Frontier

The past 3 years have witnessed a revolution in genome editing afforded by CRISPR/Cas9. This bacterial immune system consists of an RNA-guided DNA nuclease (Cas9), whose activity can easily be programmed to target specific host DNA sites by sequence embedded within a 20 nucleotide "guide" RNA species. The term "sgRNA" refers to an engineered single guide RNA commonly used for experimental purposes.[37] Expression of two components, Cas9 and sgRNA, is necessary and sufficient to this technology to a wide variety of mammalian cellular targets.

While multiple potential applications for CRISPR/Cas9 in experimental sciences have been described, here we will focus on its potential utility for functional genomic screening.[70] To date, several large-scale sgRNA libraries have been designed containing multiple sgRNA sequences targeting all human or mouse-coding genes in lentiviral expression delivery systems. A popular experimental workflow (Fig. 6.6) has been designed to produce populations of heterogeneous lentiviralparticles containing all possible perturbations.

Therefore, large numbers of cells can be infected in a "bulk" (rather than arrayed) format, and cells possessing rare phenotypes of interest isolated by positive selection. This screening workflow significantly reduces complexity and cost of screening efforts. Genomic DNA can be isolated from cells possessing the phenotype of interest, and sgRNA sequences present in the host cell genome determined by next generation sequencing.

The experimental approach was first applied to identify genes whose absence confers resistance to chemotherapeutic agents in cancer cells.[71,72] More recently, reports have emerged using loss-of-function CRISPR screening to identify genes required for toxin-induced cell death, and in vivo in models of hematologic malignancies.[73–75] Considerable excitement exists in the functional genomic community regarding the superiority of CRISPR-based loss-of-function screening modalities above traditional RNAi approaches. However, concerns regarding possible off-target activity (DNA cleavage at sites not perfectly targeted by the 20 nucleotide sgRNA sequence) and variable on-target activity (variable mutagenic activity across

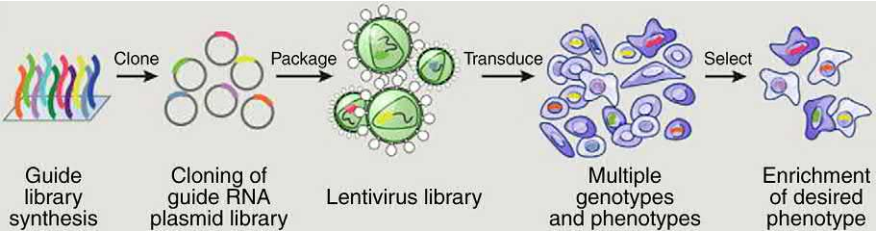

FIGURE 6.6 Workflow for functional genomic screening using CRISPR/Cas9. *Source: Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering.* Cell *2014;***157***(6):1262–78.*

multiple sgRNAs) remain. Current efforts to minimize off-target[76,77] and maximize on-target cleavage[78] are likely to further improve the performance of this exciting functional genomic screening approach.

The use of a modified, catalytically inactive Cas9 to modulate gene expression is an additional possibility afforded by CRISPR/Cas9. Here, a nuclease-dead form of Cas9 can be fused to a potent activating or inhibitory domain. This artificial TF can then be recruited to target gene promoter sequences by complementary sgRNAs.[79,80] To date, genome-wide screening efforts using this approach have been limited, but this represents an exciting complement to existing RNAi and cDNA-based approaches to modulate (rather than eliminate) target gene expression.

5 OUTLOOK

Understanding the landscape of regulatory elements of cells important for bone and mineral homeostasis is still in its infancy. More comprehensive genome-wide maps of histone modifications and TF binding sites are needed. For example, although "master regulator" TFs that control chondrocyte, osteoblast, and osteoclast differentiation are known, osteocyte-specific TFs responsible for osteocytogenesis are unknown. Understanding the epigenetic changes that dictate cell stage transitions and differentiation will further contribute to knowledge that is applicable to both physiology and disease states.

To date, no genome-wide CRISPR screens have been reported in bone cells. As reviewed earlier, powerful in vitro models of osteoblast, osteoclast, and osteocyte functions exist. Combining these models to genome-wide screens carefully designed to interrogate cellular phenotypes of interest will undoubtedly lead to discovery of novel genes crucial for bone cell function.

6 SUMMARY

The current postgenomic era represents an exciting time in biomedical research. Information abounds, and rapidly new tools are being developed to discover new genes and probe their function. Combining these tools with powerful in vitro and in vivo models commonly used in skeletal biology research will lead to new discoveries and new drug targets.

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CHAPTER

7

Mouse Models: Approaches to Generate In Vivo Models for Hereditary Disorders of Mineral and Skeletal Homeostasis

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1 INTRODUCTION

In vivo models may facilitate the investigation of the pathophysiological mechanisms that underlie inherited bone disorders, as well as preclinical translational studies to assess new treatments.[1] In addition, in vivo models are of particular importance for the study of multisystem disorders, such as skeletal diseases that may affect multiple cell types or multiple tissues, and thus have advantages over in vitro cell culture methods, which are important for functional and molecular investigations, but are nevertheless isolated systems that are outside the context of the whole organism.[1] In recent years, the mouse has increasingly become the species of choice for developing such in vivo mammalian models, as it has many similar physiological systems to man.[1] The small size of mice, coupled with their ability to reproduce rapidly with a short-generation time, yields many practical advantages for studying genetic disorders, as well as the effects of different diets and environments.[1] In addition, the coding regions of mouse and human genomes share ~85% nucleotide sequence identity[2] and development of gene targeting methods in the mouse has facilitated the generation of mouse models for human disorders by using mutagenic, transgenic, and targeted approaches.[3] Finally, the availability of inbred mouse strains for these models offers two advantages: first, they help to minimize the variations in phenotype that may occur from genetic modifiers; and second, generating the same disease model in different strains conversely enables interstrain differences in phenotype to be studied, as well as identifying the influence of genetic modifiers, which may also contribute to differential phenotypes in man.

Inherited bone disorders may be associated with abnormal mineral metabolism, which may result in alterations of plasma or urine calcium or phosphate concentrations; or skeletal dysplasia that may be due to defects in structural proteins. This chapter will review the main strategies that have been used for generating mouse models (Tables 7.1 and 7.2), and illustrate their utility with some examples of mouse models that have been generated for inherited disorders of calcium metabolism and parathyroid hormone (PTH) signaling (Tables 7.3–7.5). Also summarized are details of other mouse models that have been generated for disorders of phosphate homeostasis, vitamin D metabolism, and the pathways of fibroblast growth factor 23 (FGF23) signaling (Tables 7.6 and 7.7); and skeletal dysplasias due to abnormalities of FGF receptors (FGFRs), collagens, transcription factors, and bone-specific proteins (Tables 7.8–7.10).

2 METHODS FOR GENERATING MOUSE MODELS

2.1 Nontargeted Strategies

Spontaneous mutations in mice may result in benign phenotypes, such as variable coat colors, or in disorders that have similarities to diseases in man, for example, the hyperphosphatemia (*Hyp*) mouse, which is representative of X-linked hyperphosphatemia in man.[59] Such *spontaneous mutations* occur at very-low frequencies, thus several techniques that increase the rate of mutation induction in the mouse genome by either nontargeted (random) or targeted strategies have been

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TABLE 7.1 Methods Resulting in Mutations

Methods	Examples	References
Spontaneous	Hypophosphatemic rickets (*Hyp*) mouse due to mutation of *Phex*[a]	4,6
Radiation	Gyro (*Gy*) mouse, which has hypophosphatemic rickets and hypercalciuria, due to deletion of *Phex* and spermine synthase genes on the X chromosome	6–8
Chemical	*Nuf* mouse, which has autosomal dominant hypocalcemia with cataracts due to an activating CaSR mutation, induced by an alkylating agent, isopropyl methane sulfonate (iPMS), which is mutagenic to testicular and epididymal sperm DNA[b]	9
Molecular biological	Deletion of an allele (knock out), for example, *Gcm2*[c]; introduction of point mutation (knock in), for example, *Pthr1*[d]; transgenic that overexpresses a gene, for example, *Fgf23*[e]	10–13

[a] *Phex*—Phosphate-regulating gene with homology to endopeptidases on the X chromosome.
[b] N-ethyl-N-nitrosourea (ENU) is another alkylating agent that can be used.
[c] *Gcm2, Glial cells missing homolog 2.*
[d] *Pthr1, Parathyroid hormone receptor 1.*
[e] *Fgf23, Fibroblast growth factor 23.*
Adapted from Piret SE, Thakker RV. Mouse models for inherited endocrine and metabolic disorders. J Endocrinol *2011;***211***:211–30.*[1]

TABLE 7.2 Some Methods, With Their Advantages and Disadvantages, That Are Used for Generating Mouse Models

Strategies	Methods	Types of mutations	Advantages	Disadvantages
Nontargeted (random)	Spontaneous	Point mutations, small deletions, chromosomal rearrangements	Visible phenotype, no manipulation of genome	Very-low frequency
	Radiation	Deletions, inversions, translocations, complex rearrangements	Easier mapping due to large rearrangements	Multiple genes may be affected
	Chemical (e.g., ENU)	Primarily point mutations, some small deletions; hypo- and hypermorphs	Most potent mutagen in mice; can be used for high throughput	More complex mapping, may be multiple mutations
	Molecular biological—gene trap	Knock out	Easy to map disrupted gene; can include reporter gene	Unpredictable phenotypes
Targeted[a]	"Targeted trapping"	Knock out	High-targeting frequency	Gene must be expressed in ES cells
	Knock out	Loss of function		
	Conventional	Disruption of endogenous gene	Can include reporter gene	Gene lost in every tissue; homozygotes often embryonic lethal
	Tissue specific (conditional)	Disruption of gene in tissue(s) of interest	Can mimic somatic loss of function; can overcome embryonic lethality of knock out	Requires breeding with second mouse line; some promoters have "leaky" expression in other tissues
	Inducible	Disruption of gene at time of choice	Can mimic somatic loss of function; can overcome embryonic lethality of knock out	Less than 100% knock out
	Knock in	Mutation of choice including toxic gain of function	Introduction of specific mutation	Labor intensive; expensive
	Transgenic	Activating or dominant mutations; can also inject shRNA for RNAi	Introduction of specific mutation or shRNA of interest	Random insertion into genome; transgenes often overexpressed
	Genome editing	Loss of function or mutation of choice including toxic gain of function	More efficient than conventional knock out and can introduce specific mutation	Potential for off-target effects

[a] *All the targeted methods use molecular biological mutagenesis (Table 7.1). Table compiled from Refs. 3,14–21.*
Adapted from Piret SE, Thakker RV. Mouse models for inherited endocrine and metabolic disorders. J Endocrinol *2011;***211***:211–30.*[1]
ENU, *N*-ethyl-*N*-nitrosourea; ES, embryonic stem; RNAi, RNA inhibition; shRNA, short-hairpin RNA.

TABLE 7.3 Some In Vivo Mouse Models for Inherited Hypoparathyroid Disorders Associated With Hypocalcemia

Diseases (OMIM)	Gene defects	Proteins	Mouse models	Heterozygous mouse phenotypes	Homozygous mouse phenotypes	References
DiGeorge syndrome type 1 (188400)	*TBX1* loss of function	T-box 1	Conventional *Tbx1* knock out	Cardiac outflow tract abnormalities	Thymic, parathyroid hypoplasia; abnormal facial structures and cleft palate; cardiac OFT abnormalities; absent hyoid bone; delayed cranial bone ossification, clavicular hypoplasia	22–25
			Pharyngeal endoderm specific *Tbx1* knock out	No phenotype	Neonatal death; absent middle and outer ear; craniofacial abnormalities; absent thymus and parathyroid; thyroid hypoplasia; cardiac OFT abnormalities	26,27
			Otic vesicle specific *Tbx1* knock out	ND	Abnormal inner ear development (complete absence in 75%)	27
			Mesoderm specific *Tbx1* knock out	ND	Malformation of inner ear; proximal mandible defects; thyroid hypoplasia; cardiovascular defects; delayed cranial bone ossification	25,28–31
			Osteoprogenitor specific *Tbx1* knock out	ND	Perinatal death; failure of hyoid bone ossification; small parietal bones; hypoplastic clavicle	25
			Neural crest specific *Tbx1* knock out	ND	Perinatal death; failure of hyoid bone ossification	25
FIH (146200)	*PTH* loss of function	Parathyroid hormone	Conventional *Pth* knock out	Normal serum biochemistry	Hypocalcemia, hyperphosphatemia, enlarged parathyroids; bone abnormalities	32,33
FIH (146200)	*GCMB* loss of function	Glial cells missing homologue B	Conventional *Gcm2* knock out	No phenotype	Hypocalcemia, hypophosphatemia; absent parathyroids; high bone mass	10

ND, Not determined; OFT, outflow tract.

TABLE 7.4 Some In Vivo Mouse Models for Inherited Disorders of PTH Signaling

Diseases (OMIM)	Gene defects	Proteins	Mouse models	Phenotypes	References
Blomstrand's chondro-dysplasia (215045)	*PTHR1* loss of function	PTH receptor 1	Conventional knock out (*Pthr1*$^{-/-}$)	Embryonic lethal; skeletal abnormalities; excessive mineralization; delayed chondrocyte differentiation	34–36
			Chondrocyte-specific *Pthr1* knock out	Accelerated chondrocyte differentiation	37
			Inducible chondrocyte-specific *Pthr1* knock out	Shortening of long bones, chondrocyte apoptosis	38
			Inducible osteocyte-specific *Pthr1* knock out	Mild osteopenia; reduced trabecular bone; reduced BMD	39
			Constitutive osteocyte-specific *Pthr1* knock out	Normal serum calcium, phosphate and PTH; increased BMD; blunted anabolic and catabolic responses to intermittent and chronic PTH administration	40

(*Continued*)

TABLE 7.4 Some In Vivo Mouse Models for Inherited Disorders of PTH Signaling (*cont.*)

Diseases (OMIM)	Gene defects	Proteins	Mouse models	Phenotypes	References
Jansen's disease (156400)	*PTHR1* gain of function	PTH receptor 1	Transgenic chondrocyte-specific mutant His223Arg PTHR1	Reduced mineralization; shortened limbs, delayed chondrocyte differentiation	41
			Transgenic osteoblast-specific mutant His223Arg PTHR1	Increased osteoblasts, osteoclasts, increased trabecular bone volume, decreased cortical bone	42
			Transgenic osteocyte-specific mutant His223Arg PTHR1	Increased bone mass; increased bone remodeling; increased osteoblasts and osteoclasts	43
			Phosphorylation deficient PTHR1 knock in	Normocalcemia with low plasma PTH; reduced BMC in females and bone volume on low-calcium diet; normal increases in BMD after intermittent PTH	11,12,44
PHPIa (103580) and PPHP (612463)	*GNAS1* loss of function	$G_s\alpha$	Conventional knock out (*Gnas*$^{-/-}$)	Maternally inherited null allele: PTH resistance; all mice: subcutaneous ossifications	45–47
			Chondrocyte-specific *Gnas* knock out	Short limbs; domed skulls; epiphyseal and growth plate defects; ectopic cartilage	48
			Osteoblast/osteocyte-specific *Gnas* knock out	Shortened long bones, reduced trabecular bone; increased cortical bone; craniofacial abnormalities	49
			ENU-induced *Gnas* Val159Glu mutation	Maternal inheritance: hypocalcemia, hyperphosphatemia, elevated PTH; osseous heteroplasia	50,51
PHPIb (603233)	Loss of *GNAS1* imprinting		Conventional knock out of *Stx16* exons 4–6	No phenotypic abnormalities	52
			Conventional knock out of *Nesp55* DMR	Maternal inheritance: hypocalcemia, hyperphosphatemia, secondary hyperparathyroidism	53

BMC, Bone mineral content; BMD, bone mineral density; DMR, differentially methylated region; NESP55, neuroendocrine protein 55; PHPIa, pseudohypoparathyroidism type Ia; PHPIb, pseudohypoparathyroidism type Ib; PPHP, pseudopseudohypoparathyroidism; PTH; parathyroid hormone; STX16: syntaxin-16.

developed (Tables 7.1 and 7.2). An early example of non-targeted mutagenesis is provided by *irradiation*, which generated the *Gy* mouse, a second model for X-linked hypophosphatemia.[59] More recently, *chemical mutagens* have been used in large-scale mutagenesis programs. Successful agents include isopropyl methane sulfonate (iPMS) used to generate the *Nuf* mouse model with an activating calcium-sensing receptor (CaSR) mutation, and *N*-ethyl-*N*-nitrosourea (ENU) used to generate a mouse model for osteogenesis imperfecta with a collagen 1 alpha 1 (COL1A1) mutation. ENU, which is an alkylating agent that primarily introduces point mutations via transfer of the ENU alkyl group to the DNA base followed by mispairing and subsequent basepair substitution during the next round of DNA replication (Fig. 7.1A), is the most potent mutagen in mice.[14] Intraperitoneal injections of ENU to male mice generate approximately one mutation per 1–1.5 Mbp of sperm DNA,[14] which allows the mutations to be inherited (Fig. 7.1B). ENU mutagenesis programs utilize two complementary approaches, which are *phenotype-driven* and *genotype-driven* screens. In phenotype-driven screens, the offspring of mutagenized mice are assessed for phenotypic variances, using a panel of morphological, biochemical, or behavioral tests, in a "hypothesis-generating" strategy, which aims to elucidate new genes, pathways, and mechanisms for a disease phenotype[14] (Fig. 7.1B). By establishing appropriate matings, phenotype-driven screens can be used to identify dominant or recessive phenotypes. Genotype-driven screens, in which mutations in a gene of interest are sought, are "hypothesis driven" and are feasible by using available parallel archives of DNA and

TABLE 7.5 Mouse Models for Hypercalcemic and Hypocalcemic Disorders Due to CaSR Mutations

Model types	Heterozygous phenotypes	Homozygous phenotypes	References
Loss-of-function			
• Conventional knock out (*Casr*$^{-/-}$)	Hypercalcemia, hypocalciuria, elevated serum PTH	Severe hypercalcemia, high serum PTH, parathyroid hyperplasia, bone abnormalities, neonatal death	54–56
• Parathyroid-specific knock out (PT-KO)	Hypercalciuria, hypercalcemia, mild hyperparathyroidism	Severe hyperparathyroidism, hypercalcemia, hypercalciuria, skeletal undermineralization, neonatal death	57
• Osteoblast-specific knock out (COL-KO)	No phenotype	Undermineralization of bone, multiple fractures	57
• Inducible chondrocyte-specific knock out (Tam-Cart-KO)	ND	Short bones	57
Gain-of-function			
• iPMS mutant (*Nuf*), Leu723Gln	Hypocalcemia, hyperphosphatemia, low plasma PTH, some renal ectopic calcification	Hypocalcemia, hyperphosphatemia, low plasma PTH, widespread ectopic calcification	9
• Knock-in models: Cys129Ser, Ala843Glu	Hypocalcemia, hyperphosphatemia, low plasma PTH, low plasma 1,25$(OH)_2D_3$, hypercalciuria, renal calcification	ND	58

iPMS, Isopropyl methane sulfonate; ND, not determined; PTH, parathyroid hormone.
Adapted from Piret SE, Thakker RV. Mouse models for inherited endocrine and metabolic disorders. J Endocrinol *2011;***211***:211–30.*[1]

TABLE 7.6 Some In Vivo Mouse Models for Inherited Bone Disorders With Abnormalities of Phosphate, Vitamin D, and FGF23

Diseases (OMIM)	Gene defects	Proteins	Mouse models	Phenotypes	References
HHRH (241530)	*NPT2C* loss of function	Sodium-dependent phosphate transporter 2c	Conventional *Npt2c* knock out	Hypercalcemia, hypercalciuria, high 1,25$(OH)_2D$, no phosphate or bone phenotype	[a]
			Inducible renal epithelia-specific *Npt2c* knock out	Normal serum calcium, phosphate, PTH, FGF23, and 1,25$(OH)_2D$; normal urinary calcium excretion	[b]
			Conventional *Npt2a* knock out	Hypophosphatemia, hypercalcemia, hypercalciuria, no rickets	[c]
			Double *Npt2c*/*Npt2a* knock out	Severe hypophosphatemia, hypercalcemia, high 1,25$(OH)_2D$, rickets	[d]
VDDR1A (264700)	*CYP27B1* loss of function	25(OH)D 1-α-hydroxylase	Conventional *Cyp271b* knock out	Hypophosphatemia, hypocalcemia, secondary hyperparathyroidism, rickets, defective cartilage mineralization	[e,f]
			Chondrocyte-specific *Cyp27b1* knock out	Widened growth plate hypertrophic zones, reduced osteoclastogenesis, increased bone volume	[g]
VDDR2A (277440)	*VDR* loss of function	Vitamin D receptor	Conventional *Vdr* knock out	Hypophosphatemia, hypocalcemia, secondary hyperparathyroidism, rickets, defective growth plates	[h,i]
			Intestine-specific *Vdr* knock out	Normocalcemia, normo-phosphatemia, elevated serum PTH and 1,25$(OH)_2D$, normal bone growth but reduced postweaning bone mass accrual, spontaneous fractures	[j]
			Osteoblast-specific *Vdr* knock out	Normal serum calcium, phosphate, PTH, 1,25$(OH)_2D$, FGF23; increased BMD and trabecular bone volume; decreased bone resorption	[k]

(*Continued*)

TABLE 7.6 Some In Vivo Mouse Models for Inherited Bone Disorders With Abnormalities of Phosphate, Vitamin D, and FGF23 (*cont.*)

Diseases (OMIM)	Gene defects	Proteins	Mouse models	Phenotypes	References
			Double *Vdr*/*Pth* knock out	Severe hypocalcemia; hyperphosphatemia, shortened long bones, reduced trabecular bone volume, decreased chondrocyte proliferation	[l]
ADHR (193100)	*FGF23* gain of function	Fibroblast growth factor 23	Transgenic mutant Arg176Gln FGF23	Hypophosphatemia due to renal phosphate wasting, secondary hyperparathyroidism, rickets, osteomalacia, decreased bone resorption	[m]
Diseases	**Gene defects**	**Proteins**	**Mouse models**	**Phenotypes**	**References**
			Transgenic osteoblast-specific FGF23 overexpression	Hypophosphatemia due to renal phosphate wasting, secondary hyperparathyroidism, rickets	[n]
			Fgf23$^{-/-}$/osteoblast-specific FGF23 overexpression double mutants	Hypophosphatemia, secondary hyperparathyroidism, rickets	[o]
XLHR (307800)	*PHEX* loss of function	PHEX	Spontaneous *Phex* deletions (hyp, hyp-Duk, hyp-2J)	Hypophosphatemia due to renal phosphate wasting, short limbs, kyphosis, rickets, osteomalacia	4–6,[p]
			Induced *Phex* mutations (Gy, SkaI, Pug, Jrt)	Hypophosphatemia due to renal phosphate wasting, short limbs, rickets, osteomalacia	[q–t]
			Osteoblast/osteocyte-specific *Phex* knock out	Hypophosphatemia due to renal phosphate wasting, short limbs, rickets, osteomalacia	[u]
			Hyp/ubiquitous PHEX overexpression	Hypophosphatemia due to renal phosphate wasting, partial correction of Hyp rickets and low BMD	[v]
			Hyp/osteoblast-specific PHEX overexpression	Hypophosphatemia due to renal phosphate wasting, partial correction of low BMD	[w,x]
			Hyp/*Pth*$^{-/-}$	Early postnatal lethality, serum phosphate levels and skeletal defects similar to *Pth*$^{-/-}$	[y]
ARHR (241520)	*DMP1* loss of function	Dentin matrix protein 1	*Dmp1*$^{-/-}$	Hypophosphatemia due to renal phosphate wasting, defective bone mineralization, rickets, defective cartilage formation, short limbs	[z–bb]
			Dmp1$^{-/-}$/osteoblast-specific DMP1 overexpression	Hypophosphatemia, rickets, defective mineralization of *Dmp1*$^{-/-}$ mice all corrected	[cc]
			Dmp1$^{-/-}$/knock in with cleavage-defective DMP1	Failure to rescue *Dmp1*$^{-/-}$ phenotype	[dd]
TC/HHS (211900)	*FGF23* loss of function	Fibroblast growth factor 23	Conventional *Fgf23* knock out	Hyperphosphatemia, elevated 1,25$(OH)_2$D, increased BMC, decreased BMD, limb deformities, mineralization of soft tissues, defective osteoblast maturation	[ee,ff]
	GALNT3 loss of function	GALNT3	Conventional *Galnt3* knock out	Hyperphosphatemia, inappropriately normal 1,25$(OH)_2$D, increased BMD in males	[gg]
			ENU-induced *Galnt3* Trp589Arg mutation	Hyperphosphatemia, ectopic calcification, low serum FGF23, elevated serum 1,25$(OH)_2$D, increased BMD, cortical bone and trabecular number	[hh]
	KL loss of function	Klotho	Conventional *Kl* knock out	Kyphosis, soft tissue calcification, osteopenia, hyperphosphatemia, hypercalcemia, elevated serum 1,25$(OH)_2$D and FGF23	[ii,jj]
			Renal distal tubule-specific *Kl* partial knock out	Hyperphosphatemia, elevated serum FGF23, decreased serum PTH, normal serum 1,25$(OH)_2$D, normocalcemia, hypercalciuria	[kk]

TABLE 7.6 Some In Vivo Mouse Models for Inherited Bone Disorders With Abnormalities of Phosphate, Vitamin D, and FGF23 (*cont.*)

Diseases	Gene defects	Proteins	Mouse models	Phenotypes	References
			Parathyroid-specific *Kl* knock out	Normophosphatemia, normocalcemia, normal serum PTH and FGF23, elevated serum $1,25(OH)_2D$, normal BMD	[ll]

[a] *Segawa H, Onitsuka A, Kuwahata M, et al. Type IIc sodium-dependent phosphate transporter regulates calcium metabolism.* J Am Soc Nephrol *2009;***20***:104–13.*
[b] *Myakala K, Motta S, Murer H, et al. Renal-specific and inducible depletion of NaPi-IIc/Slc34a3, the cotransporter mutated in HHRH, does not affect phosphate or calcium homeostasis in mice.* Am J Physiol Renal Physiol *2014;***306***:F833–43.*
[c] *Beck L, Karaplis AC, Amizuka N, Hewson AS, Ozawa H, Tenenhouse HS. Targeted inactivation of Npt2 in mice leads to severe renal phosphate wasting, hypercalciuria, and skeletal abnormalities.* Proc Natl Acad Sci USA *1998;***95***:5372–7.*
[d] *Segawa H, Onitsuka A, Furutani J, et al. Npt2a and Npt2c in mice play distinct and synergistic roles in inorganic phosphate metabolism and skeletal development.* Am J Physiol Renal Physiol *2009;***297***:F671–8.*
[e] *Dardenne O, Prud'homme J, Arabian A, Glorieux FH, St-Arnaud R. Targeted inactivation of the 25-hydroxyvitamin D(3)-1(alpha)-hydroxylase gene (CYP27B1) creates an animal model of pseudovitamin D-deficiency rickets.* Endocrinology *2001;***142***:3135–41.*
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[h] *Li YC, Pirro AE, Amling M, et al. Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia.* Proc Natl Acad Sci USA *1997;***94***:9831–5.*
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[n] *Larsson T, Marsell R, Schipani E, et al. Transgenic mice expressing fibroblast growth factor 23 under the control of the alpha1(I) collagen promoter exhibit growth retardation, osteomalacia, and disturbed phosphate homeostasis.* Endocrinology *2004;***145***:3087–94.*
[o] *DeLuca S, Sitara D, Kang K, et al. Amelioration of the premature ageing-like features of Fgf-23 knockout mice by genetically restoring the systemic actions of FGF-23.* J Pathol *2008;***216***:345–55.*
[p] *Lorenz-Depiereux B, Guido VE, Johnson KR, et al. New intragenic deletions in the Phex gene clarify X-linked hypophosphatemia-related abnormalities in mice.* Mamm Genome *2004;***15***:151–61.*
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[t] *Owen C, Chen F, Flenniken AM, et al. A novel* Phex *mutation in a new mouse model of hypophosphatemic rickets.* J Cell Biochem *2012;***113***:2432–41.*
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[cc] *Lu Y, Yuan B, Qin C, et al. The biological function of DMP-1 in osteocyte maturation is mediated by its 57-kDa C-terminal fragment.* J Bone Miner Res *2011;***26***:331–40.*
[dd] *Sun Y, Prasad M, Gao T, et al. Failure to process dentin matrix protein 1 (DMP1) into fragments leads to its loss of function in osteogenesis.* J Biol Chem *2010;***285***:31713–22.*
[ee] *Sitara D, Razzaque MS, Hesse M, et al. Homozygous ablation of fibroblast growth factor-23 results in hyperphosphatemia and impaired skeletogenesis, and reverses hypophosphatemia in Phex-deficient mice.* Matrix Biol *2004;***23***:421–32.*
[ff] *Shimada T, Kakitani M, Yamazaki Y, et al. Targeted ablation of Fgf23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism.* J Clin Invest *2004;***113***:561–8.*
[gg] *Ichikawa S, Sorenson AH, Austin AM, et al. Ablation of the Galnt3 gene leads to low-circulating intact fibroblast growth factor 23 (Fgf23) concentrations and hyperphosphatemia despite increased Fgf23 expression.* Endocrinology *2009;***150***:2543–50.*
[hh] *Esapa CT, Head RA, Jeyabalan, et al. A mouse with an* N*-ethyl-*N*-nitrosourea (ENU) induced Trp589Arg Galnt3 mutation represents a model for hyperphosphatemic familial tumoural calcinosis.* PLoS One *2012;***7***:e43205.*
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[jj] *Yuan Q, Sato T, Densmore M, et al. Deletion of PTH rescues skeletal abnormalities and high osteopontin levels in Klotho−/− mice.* PLoS Genet *2012;***8***:e1002726.*
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ADHR, Autosomal dominant hypophosphatemic rickets; ARHR, autosomal recessive hypophosphatemic rickets; VDDR: vitamin D-dependent rickets; GALNT3, UDP-*N*-acetyl-alpha-D-galactosamine: polypeptide *N*-acetylgalactosaminyl transferase-3; HHRH, hereditary hypophosphatemic rickets with hypercalciuria; $1,25(OH)_2D$, 1,25-dihydroxy-vitamin D; XLHR, X-linked hypophosphatemic rickets.

TABLE 7.7 Some Compound Mouse Mutants With Disruption of Phosphate, Vitamin D and FGF23 Signaling

Mouse models	Phenotypes	References
Hyp/Dmp1$^{-/-}$	Phenotypes of growth retardation, increased serum FGF23, increased serum PTH, hypophosphatemia, and reduced BMD no more severe than single mutants	[a]
Hyp/Galnt3$^{-/-}$	Phenotypes of growth retardation, hypophosphatemia less severe than in *Phex* mice	[b]
Fgf23$^{-/-}$*/hyp*	Dominance of *Fgf23*$^{-/-}$ phenotype over hyp phenotype: hyperphosphatemia, skeletal deformities similar to *Fgf23*$^{-/-}$ mice	[c,d]
Fgf23$^{-/-}$*/Dmp1*$^{-/-}$	Dominance of Fgf23 null phenotype over Dmp1 null phenotype: hyperphosphatemia, elevated 1,25$(OH)_2$D levels, growth plate abnormalities, no rickets	[e]
Fgf23$^{-/-}$*/Npt2a*$^{-/-}$	Hypophosphatemia, high 1,25$(OH)_2$D, skeletal phenotype similar to *Fgf23*$^{-/-}$ mice	[f]
Fgf23$^{-/-}$*/Cyp27b1*$^{-/-}$	Hypophosphatemia, secondary hyperparathyroidism, decreased BMC and BMD, reversal of soft tissue mineralization in *Fgf23*$^{-/-}$ mice, rickets	[g]
Fgf23$^{-/-}$*/Vdr*$^{-/-}$	Normophosphatemia, bone phenotype identical to that in *Vdr*$^{-/-}$ mice, no soft tissue mineralization	[h]
Fgf23$^{-/-}$*/Kl*$^{-/-}$	Similar to *Kl*$^{-/-}$ alone: hyperphosphatemia, elevated 1,25$(OH)_2$D (similar levels to *Fgf23*$^{-/-}$ and *Kl*$^{-/-}$ mice), soft tissue and vascular calcification	[i]
Fgf23$^{-/-}$*/Pth*$^{-/-}$	Further increase in serum phosphate compared to *Fgf23*$^{-/-}$ alone and *Pth*$^{-/-}$ alone; normocalcemia; lower 1,25$(OH)_2$D than *Fgf23*$^{-/-}$ alone; partially rescued bone phenotypes compared to *Fgf23*$^{-/-}$ alone	[j]
Kl$^{-/-}$*/Fgf23*^{R176Q}	Dominance of *Kl*$^{-/-}$ phenotype over FGF23 gain of function: hyperphosphatemia, elevated 1,25$(OH)_2$D, increased skeletal mineralization, soft tissue mineralization	[k]
Kl$^{-/-}$*/hyp*	Hyperphosphatemia, elevated 1,25$(OH)_2$D (similar level to *Kl*$^{-/-}$ mice), dense bone mineralization, soft tissue and vascular calcification	[l,m]
Kl$^{-/-}$*/Dmp1*$^{-/-}$	Growth retardation and survival improved compared to *Kl*$^{-/-}$ alone; osteomalacia and rickets improved, and bone remodeling defect corrected compared to *Dmp1*$^{-/-}$ alone; similar serum biochemistry to *Kl*$^{-/-}$ alone, except for FGF23 level between *Dmp1*$^{-/-}$ and *Kl*$^{-/-}$ alone	[n]
Kl$^{-/-}$*/Npt2a*$^{-/-}$	Mild hypophosphatemia, elevated 1,25$(OH)_2$D, reduction of soft tissue calcification usually found in *Kl*$^{-/-}$ mice	[o]
Kl$^{-/-}$*/Cyp27b1*$^{-/-}$	Dominance of *Cyp27b1*$^{-/-}$ phenotype over *Kl*$^{-/-}$ phenotype: hypophosphatemia, secondary hyperparathyroidism, absence of ectopic calcification	[p]
Kl$^{-/-}$*/Pth*$^{-/-}$	Normocalcemia; further elevation in serum phosphate compared to *Kl*$^{-/-}$ and *Pth*$^{-/-}$ alone; soft tissue calcification similar to *Kl*$^{-/-}$ alone; slightly reduced serum 1,25$(OH)_2$D and FGF23 levels compared to *Kl*$^{-/-}$ alone; bone phenotypes of *Kl*$^{-/-}$ mice rescued	[q]
Kl$^{-/-}$*/Vdr*$^{-/-}$	Normocalcemia, normophosphatemia; bone phenotypes of *Kl*$^{-/-}$ mice rescued	[r]
Hyp/Fgfr1$^{Dmp\text{-}cKO}$	Growth retardation, rickets and increased serum FGF23 less severe than in *Hyp*; hypophosphatemia, elevated PTH, and elevated 1,25$(OH)_2$D in *Hyp* restored to normal	[s]
Hyp/Fgfr3$^{-/-}$	Growth retardation; kyphosis; elevated FGF23 (twice that in *Hyp* mice); hypophosphatemia; normal 1,25$(OH)_2$D; bowing of long bones; rickets; osteomalacia; bone mineralization defect, that is, no rescue of *Hyp* phenotype	[t]
Hyp/Fgfr4$^{-/-}$	Growth retardation; kyphosis; elevated FGF23 (twice that in *Hyp* mice); hypophosphatemia; normal 1,25$(OH)_2$D; bowing of long bones; rickets; osteomalacia; bone mineralization defect, that is, no rescue of *Hyp* phenotype	[t]
Fgfr1^{MM}*/Fgfr4*$^{-/-}$	Hyperphosphatemia, elevated serum FGF23 and 1,25$(OH)_2$D, decreased serum PTH, incomplete suppression of serum FGF23 levels on low-phosphate diet	[u]
Fgfr3$^{-/-}$*/Fgfr4*$^{-/-}$	Small size; early death; elevated 1,25$(OH)_2$D; elevated FGF23; no reduction in 1,25$(OH)_2$D after administration of FGF23, but decreased serum PTH and increased serum phosphorous	[v,w]

TABLE 7.7 Some Compound Mouse Mutants With Disruption of Phosphate, Vitamin D and FGF23 Signaling (*cont.*)

Mouse models	Phenotypes	References
Hyp/*Fgfr3*$^{-/-}$/*Fgfr4*$^{-/-}$	Small size; kyphosis; partial correction of *Hyp* rickets and low serum phosphate. Overcorrection of *Hyp* phenotype with respect to 1,25(OH)$_2$D. Further increase in *Hyp* elevated serum FGF23	[x]

[a] *Martin A, Liu S, David V, et al. Bone proteins PHEX and DMP1 regulate fibroblastic growth factor Fgf23 expression in osteocytes through a common pathway involving FGF receptor (FGFR) signaling.* FASEB J *2011;***25***:2551–62.*
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[c] *Sitara D, Razzaque MS, Hesse M, et al. Homozygous ablation of fibroblast growth factor-23 results in hyperphosphatemia and impaired skeletogenesis, and reverses hypophosphatemia in Phex-deficient mice.* Matrix Biol *2004;***23***:421–32.*
[d] *Liu S, Zhou J, Tang W, Jiang X, Rowe DW, Quarles LD. Pathogenic role of Fgf23 in Hyp mice.* Am J Physiol Endo Metab *2006;***291***:E38–49.*
[e] *Liu S, Zhou J, Tang W, Menard R, Feng JQ, Quarles LD. Pathogenic role of Fgf23 in Dmp1-null mice.* Am J Physiol Endo Metab *2008;***295***:E254–61.*
[f] *Sitara D, Kim S, Razzaque MS, et al. Genetic evidence of serum phosphate-independent functions of FGF-23 on bone.* PLoS Genet *2008;***4***:e1000154.*
[g] *Sitara D, Razzaque MS, St-Arnaud R, et al. Genetic ablation of vitamin D activation pathway reverses biochemical and skeletal anomalies in Fgf-23-null animals.* Am J Pathol *2006;***169***:2161–70.*
[h] *Hesse M, Frohlich LF, Zeitz U, Lanske B, Erben RG. Ablation of vitamin D signaling rescues bone, mineral, and glucose homeostasis in Fgf-23 deficient mice.* Matrix Biol *2007;***26***:75–84.*
[i] *Nakatani T, Sarraj B, Ohnishi M, et al. In vivo genetic evidence for klotho-dependent, fibroblast growth factor 23 (Fgf23)-mediated regulation of systemic phosphate homeostasis.* FASEB J *2009;***23***:433–41.*
[j] *Yuan Q, Sitara D, Sato T, et al. PTH ablation ameliorates the anomalies of Fgf23-deficient mice by suppressing the elevated vitamin D and calcium levels.* Endocrinology *2011;***152***:4053–61.*
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[m] *Brownstein CA, Zhang J, Stillman A, et al. Increased bone volume and correction of HYP mouse hypophosphatemia in the Klotho/HYP mouse.* Endocrinology *2010;***151***:492–501.*
[n] *Rangiani A, Cao Z, Sun Y, et al. Protective roles of DMP1 in high phosphate homeostasis.* PLoS One *2012;***7***:e42329.*
[o] *Ohnishi M, Nakatani T, Lanske B, Razzaque MS. In vivo genetic evidence for suppressing vascular and soft-tissue calcification through the reduction of serum phosphate levels, even in the presence of high serum calcium and 1,25-dihydroxyvitamin d levels.* Circ Cardiovasc Genet *2009;***2***:583–90.*
[p] *Ohnishi M, Nakatani T, Lanske B, Razzaque MS. Reversal of mineral ion homeostasis and soft-tissue calcification of klotho knockout mice by deletion of vitamin D 1alpha-hydroxylase. Kidney Int 2009;75:1166–72.*
[q] *Yuan Q, Sato T, Densmore M, et al. Deletion of PTH rescues skeletal abnormalities and high osteopontin levels in Klotho−/− mice.* PLoS Genet *2012;***8***:e1002726.*
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[v] *Weinstein M, Xu X, Ohyama K, Deng CX. FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung.* Development *1998;***125***:3615–23.*
[w] *Gattineni J, Twombley K, Goetz R, Mohammadi M, Baum M. Regulation of serum 1,25(OH)2 vitamin D3 levels by fibroblast growth factor 23 is mediated by FGF receptors 3 and 4.* Am J Physiol Renal Physiol *2011;***301***:F371–7.*
[x] *Li H, Martin A, David V, Quarles LD. Compound deletion of Fgfr3 and Fgfr4 partially rescues the Hyp mouse phenotype.* Am J Physiol Endo Metab *2011;***300***:E508–17.*
BMD, Bone mineral density; Dmp1, dentin matrix protein 1; FGFR, FGF receptor; *Fgfr1*^{MM}, metanephric mesenchyme-specific *Fgfr1* knock out; *Fgfr1*$^{Dmp1\text{-}cKO}$, osteocyte-specific *Fgfr1* knock out; Fgf23, fibroblast growth factor 23; Kl, klotho; Npt2a, sodium-dependent phosphate transporter 2a; PTH, parathyroid hormone; Vdr, vitamin D receptor; 1,25(OH)$_2$D, 1,25-dihydroxyvitamin D.

TABLE 7.8 Some In Vivo Mouse Models for Inherited Bone Disorders With Abnormalities of FGF Receptors 1-3 (FGFR1-3)

Genes	Mouse models	Phenotypes	References
Fgfr1	Osteochondro-specific *Fgfr1* knock out	Delayed osteoblast differentiation, increased bone mass	[c]
	Osteoblast-specific *Fgfr1* knock out	Accelerated osteoblast differentiation, increased bone mass	[c]
	Osteocyte-specific *Fgfr1* knock out	No bone phenotype, increased serum FGF23, normal serum calcium, phosphorus, PTH or 1,25(OH)$_2$D	[d]
	Bone-marrow monocyte/osteoclast-specific *Fgfr1* knock out	Increased BMD, increased trabecular bone thickness, defective osteoclast differentiation	[e]
	Fgfr1 Pro250Arg knock in (Pfeiffer syndrome mutation)	Postnatal craniofacial malformations due to premature fusion and mineralization of sutures; enhanced osteoblast proliferation and differentiation	[f]
	Various hypomorphic *Fgfr1* models	Loss of anterior digits; delayed ossification in distal phalanges; syndactyly; vertebral malformations	[g]

(*Continued*)

TABLE 7.8 Some In Vivo Mouse Models for Inherited Bone Disorders With Abnormalities of FGF Receptors 1-3 (FGFR1-3) (*cont.*)

Genes	Mouse models	Phenotypes	References
	Metanephric mesenchyme-specific *Fgfr1* knock out	Elevated serum FGF23; decrease in serum $1,25(OH)_2$ but not in serum phosphate after FGF23 administration	h
Fgfr2	Conventional *Fgfr2* splice variant IIIc knock out	Short and dome-shaped skull; abnormal suture fusion; delayed ossification of skeleton	i
	Osteoblast/chondrocyte-specific *Fgfr2* knock out	Growth retardation; dwarfism; dome-shaped skull; vertebral abnormalities; reduced BMD	j
	ENU-induced *Fgfr2* Trp290Arg mutation (Crouzon syndrome mutation)	Heterozygotes: short snout; dome-shaped skull; premature fusion of coronal sutures; defective endochondral ossification. Homozygotes: perinatal death; delayed ossification of coronal sutures; absent limbs; defective endochondral ossification	k
	Fgfr2 Ser252Trp knock in (Apert syndrome mutation)	Neonatal death; shortened and dome-shaped skull; premature fusion of coronal suture; ectopic cartilage at sutures; disorganized growth plates; reduced BMD and trabecular number; short long bones and vertebrae	l,m,nl–n
	Inducible mesoderm-specific *Fgfr2* Ser252Trp knock in	Premature fusion of coronal sutures; ectopic cartilage at sutures; normal parietal bone development; facial shortening	o
	Fgfr2 Pro253Arg knock in (Apert syndrome mutation)	Shortened and dome-shaped skull; premature fusion of coronal suture; shortened limbs	p
	Fgfr2 Cys342Tyr knock in (Crouzon/Pfeiffer syndrome mutation)	Heterozygotes: shortened and dome-shaped skull; premature fusion of coronal sutures. Homozygotes: shortened and dome-shaped skull; cleft palate; excessive bone formation	q
	Loss of *Fgfr2* exon 9 (activating mutation)	Shortened and dome-shaped skull; accelerated ossification and fusion of sutures	r
Fgfr3	Conventional *Fgfr3* knock out	Postnatal death in ~50%; kyphosis; scoliosis; curvature, overgrowth and increased thickness of femur and humerus; osteopenia; arthritis	s–w
	Fgfr3 Pro244Arg knock in (equivalent to Muenke syndrome mutation Pro250Arg)	Heterozygotes: small rounded skull in minority of males; reduced BMD; reduced cortical bone thickness. Homozygotes: small rounded skull in all males and minority of females; twisted snout in some; reduced BMD; reduced cortical bone thickness	x
	Fgfr3 Gly369Cys knock in (equivalent to ACH mutation Gly375Cys)	Heterozygotes: postnatal dwarfism; dome-shaped skull; reduced trabecular bone; reduced BMD; defects in bone matrix mineralization. Homozygotes: similar to heterozygotes but more severe	y,z
	Fgfr3 Gly374Arg knock in (equivalent to ACH mutation Gly380Arg)	Heterozygotes: small size; rounded and distorted skull; kyphosis; dwarfism; disorganized growth plates; premature synchondrosis closure	aa,bb
	Fgfr3 Ser365Cys knock in (equivalent to TD-I mutation Ser371Cys)	Heterozygotes: small dome-shaped skull; severe dwarfism; shortened long bones; disorganized growth plates; high-postnatal death	cc
	Fgfr3 Tyr367Cys knock in (equivalent to TD-I mutation Tyr373Cys)	Heterozygotes: narrow trunk; bowed and shortened long bones; macrocephaly; dwarfism; delayed ossification; defective chondrocyte proliferation and differentiation; reduced cortical thickness, trabecular thickness and trabecular number	dd–ff
	Chondrocyte-specific *Fgfr3* Tyr367Cys knock in	Dwarfism, short limbs; moderately dome-shaped skull; delayed secondary ossification; disorganized growth plates; reduced cortical thickness, trabecular thickness and trabecular number	ff
	Osteoblast-specific *Fgfr1* Tyr367Cys knock in	No dwarfism; normal limb length, skull shape, ossification, growth plates, cortical thickness, trabecular thickness and trabecular number	ff
	Fgfr3 Lys644Glu knock in[a] (equivalent to TD-II mutation Lys650Glu)	Heterozygotes: small size; short tail. Homozygotes: postnatal dwarfism; shortened long bones; shortened tail vertebrae; macrocephaly	gg

TABLE 7.8 Some In Vivo Mouse Models for Inherited Bone Disorders With Abnormalities of FGF Receptors 1-3 (FGFR1-3) (*cont.*)

Genes	Mouse models	Phenotypes	References
	Fgfr3 Lys644Glu knock in[b] (equivalent to TD-II mutation Lys650Glu)	Heterozygotes: neonatal death; decreased ossification; rounded skull; shortened, widened and bowed long bones with disorganized growth plates	[hh]
	Fgfr3 Lys644Met knock in (equivalent to SADDAN mutation Lys650Met)	Heterozygotes: death within 4 weeks in ~50%; shortened long bones; delayed secondary ossification. Homozygotes: embryonic lethal	[ii]
	Transgenic mutant Gly380Arg FGFR3	Short snout; dwarfism; shortened and bowed long bones; defective chondrocyte proliferation	[jj]

[a] *Mutant allele expressed at ~20% of wild-type allele expression.*
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ACH, Achondroplasia; BMD, bone mineral density; FGF23, fibroblast growth factor 23; SADDAN: severe achondroplasia with developmental delay and acanthosis nigricans; TD, thanatophoric dysplasia; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D.

TABLE 7.9 Some In Vivo Mouse Models for Inherited Osteochondrodysplasias Due to Loss of Function of Collagens

Diseases	Gene defects	Mouse models	Phenotypes	References
Osteogenesis imperfecta	Collagen type I, alpha 1 (COL1A1)	ENU-induced *Col1a1* mutation	Heterozygotes: high-postnatal lethality; severe bone deformitites; long bone and pelvic fractures; low BMD and BMC. Homozygotes: embryonic lethal	[a]
		Viral insertion causing *Col1a1* knock out	Heterozygotes: brittle bones; abnormal cortical bone. Homozygotes: embryonic lethal	[b,c]
		Col1a1 Gly349Cys knock in	Heterozygotes: perinatal lethality in ~50%; small size; bowed and thin limbs; undermineralization of skeleton	[d]
		Transgenic expressing *Col1a1* with large deletion	Perinatal death in some; extensive fractures of long bones and ribs; undermineralization of skull; short femurs	[e,f]
	Collagen type I, alpha 2 (COL1A2)	Spontaneous *Col1a2* single nucleotide deletion	Heterozygotes: decreased bone strength; Homozygotes: small size; multiple fractures; long bone deformities; osteopenia; reduced cortical bone; decreased BMC, BMD	[g–i]
Type II collagenopathies	Collagen type II, alpha I (COL2A1)	Radiation-induced *Col2a1* insertion–deletion	Heterozygotes: mild postnatal dwarfism; reduced ECM; osteoarthritis. Homozygotes: cleft palate; short skull; severe dwarfism; shortened long bones; disorganized growth plates; reduced ECM	[j–l]
		Spontaneous *Col2a1* missense mutation Arg1417Cys	Homozygotes: shortened trunk and long bones; short skull; disorganized growth plates; decreased bone volume and trabecular thickness	[m]
		ENU-induced *Col2a1* missense mutation Asp1469Ala	Heterozygotes: mild dwarfism; short limbs. Homozygotes: perinatal death; severe dwarfism; short limbs; pelvic hypoplasia; reduced ECM	[n]
		ENU-induced *Col2a1* missense mutation Ser1386Pro	Heterozygotes: short fore and hind limbs; decreased BMD; disorganized growth plates; delayed endochondral ossification. Homozygotes: perinatal lethality, cleft palate; short and wide long bones	[o]
		ENU-induced *Col2a1* splicing defect	Homozygotes at embryonic day 18.5: microcephaly; short mandible; short and wide long bones	[p]
		Conventional *Col2a1* knock out	Heterozygotes: minor skeletal changes. Homozygotes: perinatal lethality; no palate; small size; shortened and thickened long bones; disorganized growth plates	[q]
		Col2a1 Arg519Cys knock in	Mild dysplasia of long bones; mild osteoarthritis; short bones	[r]
		Col2a1 Gly1170Ser knock in	Heterozygotes: normal size and long bone lengths. Homozygotes: perinatal lethality; severely dwarfed; short trunk and limbs; truncated facial bones; cleft palate; delayed endochondral ossification; disorganized growth plates	[s]
		Transgenic expressing *COL2A1* with large deletion	High-perinatal death; cleft palate; cranial bulge; short and thick long bones; delayed bone mineralization; osteoarthritis	[t,u]
		Transgenic expressing *Col2a1* with exon 7 deletion	Small size; shortened long bones; reduced cartilaginous ECM; defective ossification; disorganized growth plates; disorganized collagen fibrils; cranial defects	[v,w]
		Inducible transgenic expressing *Col2a1* with Arg1192Cys mutation	Short trunk; short and wide long bones and vertebrae; disorganized growth plates; altered chondrocyte polarity; decreased chondrocyte proliferation	[x]
		Transgenic mutant Arg789Cys *Col2a1*	Neonatal death; severe dwarfism; shortened long bones; short snout; cleft palate; delayed ossification; disorganized cartilage; reduced collagen	[y]
Schmid metaphyseal chondrodysplasia	Collagen type X, alpha 1 (COL10A1)	*Col10a1* Asn617Lys knock in	Homozygotes: small size; shortened long bones; small and distorted pelvis; expanded hypertrophic zones; disrupted chondrocyte differentiation	[z]

TABLE 7.9 Some In Vivo Mouse Models for Inherited Osteochondrodysplasias Due to Loss of Function of Collagens (*cont.*)

Diseases	Gene defects	Mouse models	Phenotypes	References
		Transgenic expressing *Col10a1* with frame-shift mutation	Shortened long bones; *coxa vara*; thickened growth plate hypertrophic zones; disrupted chondrocyte differentiation	[aa]
		Conventional *Col10a1* knock out	Perinatal lethality in ~10%; dwarfism; kyphosis; small growth plates; small trabeculae; reduced BMC	[bb,cc]
Type XI collagen-opathies	Collagen type XI, alpha 1 (COL11A1)	Spontaneous *Col11a1* single nucleotide deletion	Heterozygotes: osteoarthritis after 3 months. Homozygotes: short snout; cleft palate; shortened limbs; disorganized chondrocytes;	[dd,ee]
	Collagen type XI, alpha 2 (COL11A2)	Conventional *Col11a2* knock out	Homozygotes: small size; short snout; facial defects; disorganized growth plates; thin articular cartilage	[ff]

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BMC, Bone mineral content; BMD, bone mineral density; ECM, extracellular matrix; ENU, *N*-ethyl-*N*-nitrosourea.

TABLE 7.10 Some In Vivo Mouse Models for Inherited Osteochondrodysplasias Due to Transcription Factors and Bone-Specific Proteins

Diseases	Gene defects	Mouse models	Phenotypes	References
Paget's disease of bone	RANK (*TNFRSF11A*) loss of function	Conventional *Rank* knock out	Severe osteopetrosis; shortened and club shaped long bones; defective bone resorption; lack of osteoclasts	[a,b]
		Spontaneous *Rank* 8bp deletion	Homozygotes: retarded growth; craniofacial defects; increased BMD; progressive kyphosis; enlarged epiphyseal growth plates; lack of osteoclasts	[c]
	Sequestosome 1 (SQSTM1) mutations	*Sqstm1* Pro394Leu knock in	Age-related osteolytic lesions in long bones; increased bone turnover; abnormal osteoclast morphology	[d]
Juvenile Paget's disease	Osteoprotegerin (OPG/*TNFRSF11B*) loss of function	Conventional *Opg* knock out	Homozygotes: postnatal death in some; spontaneous fractures; severe osteoporosis; kyphosis	[e,f]
Hereditary multiple exostoses	Exostosin 1 (EXT1) loss of function	Conventional *Ext1* knock out	Heterozygotes: increased chondrocyte proliferation. Homozygotes: embryonic lethal	[g,h]
		Chimeric chondrocyte-specific *Ext1* knock out	Multiple osteochondromas with 100% penetrance on most endochondral bones; bowing of radius	[i,j]
		Limb bud mesenchyme-specific *Ext1* knock out	Heterozygotes: no phenotype. Homozygotes: perinatal death; short and wide long bones; fused forelimb bones	[k]
		Inducible cartilage-specific *Ext1* knock out	Growth retardation; disorganized growth plates; delayed secondary ossification; increased osteoclast numbers; exostotic outgrowths	[l]
	Exostosin 2 (EXT2) loss of function	Conventional *Ext2* knock out	Heterozygotes: occasional exostoses on ribs. Homozygotes: early embryonic lethality	[m]
Hypophosphatasia	Tissue nonspecific alkaline phosphatase (ALPL) loss of function	Conventional *Alpl* knock out	Homozygotes: early postnatal death; small size; epileptic seizures in ~50%; osteopenia; spontaneous fractures; short growth plates; defective secondary ossification; craniofacial defects; craniosynostosis	[n–r]
		ENU-induced *Alpl* splice site mutation	Age-related accumulation of uncalcified osteoid in subchondral bone; ossification of joint capsules	[s]
Fibrodysplasia ossificans progressiva (FOP)	Activin A receptor, type 1 (ACVR1) gain of function	Inducible muscle-specific *Acvr1* Gln207Asp knock in	Within 20 days of induction: severely decreased mobility; loss of joint flexion; bony calluses fusing with bones; heterotopic ossification	[t]
		Chimeric *Acvr1* Arg206His knock in	Short first metatarsal; extraskeletal bone formation; heterotopic ossification in skeletal muscle and progressive immobility after injury	[u]
Diastrophic dysplasia (DTD)	DTD sulfate transporter (DTDST/SLC26A2) loss of function	*Dtdst* Ala286Val knock in	Homozygotes: reduced postnatal growth; kyphosis; early death in ~50%; shortened long bones; bite overclosure; hip dysplasia	[v]
Multiple epiphyseal dysplasia	Cartilage oligo-meric matrix protein (COMP) dominant negative mutations	*Comp* Thr583Met knock in	Disorganized growth plates; enlarged proliferative zone. After 9 weeks of age: small size; shortened long bones; hip dysplasia. At 16 months of age: articular degeneration.	[w]
		Comp Asp469Del knock in	Homozygotes: progressive short-limb dwarfism and hip dysplasia after 9 weeks of age; disorganized growth plates with hypocellularity and mislocalization of cartilage structural proteins	[x]
Chondro-dysplasia punctate (X-linked dominant)	Delta(8)-delta(7) sterol isomerase emopamil-binding protein (EBP) loss of function	Radiation induced *Ebp* Arg107Gly mutation	Heterozygous females: small size; domed skull; short snout. Hemizygous males: small size; domed skull; short limbs; cleft palate; embryonic death	[y]

TABLE 7.10 Some In Vivo Mouse Models for Inherited Osteochondrodysplasias Due to Transcription Factors and Bone-Specific Proteins (*cont.*)

Diseases	Gene defects	Mouse models	Phenotypes	References
Trichorhino-phalangeal syndrome type 1 (TRPS1)	TRPS1 loss of function	Targeted deletion of *Trps1* zinc finger domain	Heterozygotes: arched palate; kyphoscoliosis; reduced bone volume. Homozygotes: perinatal death; scoliosis; delayed ossification	z
		Conventional *Trps1* knock out	Homozygotes: perinatal death due to respiratory distress; growth retardation; shortened limb long bones; abnormal sternum and rib cage; shortened maxillary and mandibular bones; reduced chondrocyte numbers in growth plates; abnormal temporomandibular joints	aa,bb
Grebe chondrodysplasia	GDF5 loss of function	Five spontaneous *Gdf5* frame-shift deletions	Homozygotes: slightly shortened long bones; severely shortened digits; altered digit patterning	cc–ee
Cleidocranial dysplasia	Runt-related transcription factor 2 (RUNX2) loss of function	Conventional *Runx2* knock out	Heterozygotes: hypoplastic clavicles and nasal bones; reduced ossification Homozygotes: immediate perinatal death; dwarfism; short limbs; very little or no ossification.	ff,gg
		Runx2 Phe377Stop knock in	Heterozygotes: missing clavicles. Homozygotes: late embryonic death; complete loss of endochondral and intramembraneous ossification	hh
		Hypomorphic *Runx2* mutant	Heterozygotes (79%–84% expression): no phenotype. Homozygotes (55%–70% expression): reduced calvarial ossification; cranial defects; hypoplastic clavicles	ii
		Runx2-II isoform-specific knock out	Heterozygotes: osteopenia. Homozygotes: normal intramembraneous ossification; absence of endochondral ossification	jj
		Runx2-I isoform-specific hypomorph	Homozygotes: perinatal death within 24 h; diminished bone formation; absent clavicle and occipital bone; decreased trabecular number and increased trabecular separation.	kk
		Chondrocyte-specific deletion of *Runx2* exon 8	Immediate postnatal death due to respiratory failure; absent vertebrae, pelvic, scapula and metacarpal/metatarsal bones; short ribs and limb bones; craniofacial bones poorly developed or absent	ll
		Chondrocyte-specific deletion of *Runx2* exon 8	Perinatal death; domed skull, short snout, small limbs; reduced ossification; disorganized growth plates.	mm
		Osteoblast-specific deletion of *Runx2* exon 8	Comparable size and weight to wild-type mice; normal skeletal patterning; normal long bone length; normal growth plates; decreased calcification of long bones, digits and vertebrae; impaired cranial development; retarded postnatal growth; decreased bone stiffness; decreased trabecular volume, number and thickness.	nn
Campomelic dysplasia	SRY-box 9 (SOX9) loss of function	Conventional *Sox9* knock out	Heterozygotes: perinatal death; cleft palate; bent long bones and pelvic bones; defective endochondral ossification; hypoplastic scapulae	oo
		Chondrocyte-specific *Sox9* knock out	Late embryonic death; round head; short snout; short trunk and limbs; reduced endochondral ossification	pp

(*Continued*)

TABLE 7.10 Some In Vivo Mouse Models for Inherited Osteochondrodysplasias Due to Transcription Factors and Bone-Specific Proteins (*cont.*)

Diseases	Gene defects	Mouse models	Phenotypes	References
		Limb bud mesenchyme-specific *Sox9* knock out	Perinatal death; short limbs; absence of limb bones; absence of osteoblasts in limb buds	[pp]

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RANK, Receptor activator of NF-kappaB; TNFRSF11, tumor necrosis factor receptor superfamily member 11.

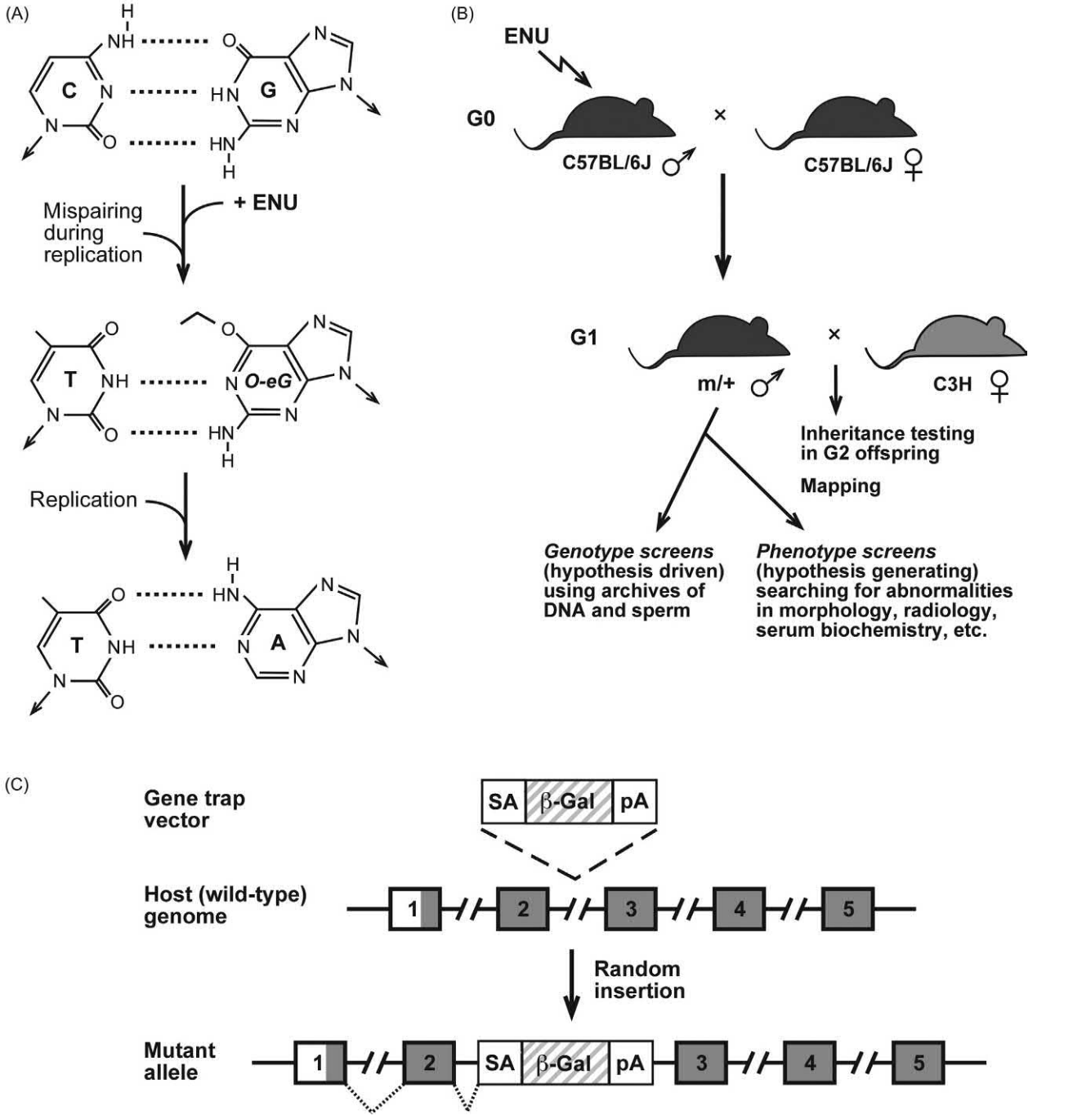


FIGURE 7.1 Methods for nontargeted (random) mutagenesis. (A) Chemical mutagenesis using *N*-ethyl-*N*-nitrosourea (ENU). ENU is an alkylating agent that transfers its ethyl group to one of a number of reactive sites on DNA nucleotides, including the O6 of guanine as shown. Modification of guanine with the ethyl group to produce O^6-ethylGuanine (O-eG), causes mispairing during DNA replication, for example, at spermatogenesis, and during subsequent replication a mutation is introduced. (B) ENU-mutagenized G0 male mice, harboring induced mutations in their sperm DNA, are mated with wild-type females of the same strain to generate G1 mice. G1 males are examined for phenotypic abnormalities (i.e., the phenotype-driven screens). Males with phenotypic abnormalities of interest are then mated with wild-type females to facilitate inheritance testing and genetic mapping in affected offspring (G2) to identify the mutation causing the phenotypic abnormality. DNA and sperm from all the G1 males are also archived to facilitate genotype-driven screens. m, Mutant allele; +, wild-type allele. (C) Insertion mutagenesis using gene trap vectors. Gene trap vectors consist of a strong splice acceptor (*SA*), a reporter gene, such as β-galactosidase (β-gal) and a polyadenine tract (pA). The gene trap randomly inserts into the host genome (dashed lines), and during splicing, the SA is used in preference to the normal genomic splice sites (splicing pattern shown by dotted lines). Filled/striped boxes denote coding sequences and open boxes denote noncoding sequences. *Source: Reproduced from Piret SE, Thakker RV. Mouse models for inherited endocrine and metabolic disorders.* J Endocrinol *2011;***211***:211–30.*[1]

sperm samples from mutagenized male mice (Fig. 7.1B). Archived DNA samples from the mutagenized male mice are used to search for mutations in the gene of interest, and once mutations are identified in the mouse DNA, then the corresponding sperm sample for the male mouse harboring the mutation is used to establish progeny carrying the mutation by in vitro fertilization.[14] It is estimated that the probability of finding three or more mutant alleles in an archive of >5000 DNA samples is >90%.[60] Thus, the gene-driven approach can be used to generate an "allelic series" of mutations within one gene, which may yield insights into genotype–phenotype correlations in the gene and disease of interest.[61]

ENU mutations most frequently result in missense mutations (>80%) that may generate hypo- and hypermorphs, although occasionally nonsense or frame-shift mutations (<10%) generating knock-out models may be obtained.[62] However, a more recent and reliable method for generating nontargeted knock-out models on a large scale is by the use of insertional mutagenesis, utilizing *gene-trap* strategies.[63,64] Gene-trap vectors usually consist of a reporter gene, either with or without a promoter, and a strong splice acceptor (SA) site, which causes any upstream exons to splice directly to the gene trap[15] (Fig. 7.1C). The vector is either electroporated or retrovirally infected into embryonic stem (ES) cells, after which it randomly inserts into the genome. Mutagenized ES cells are then reintroduced into developing blastocysts to generate chimeric mice, from which germline mutant mice can be bred (Fig. 7.2). A recent refinement of the gene-trap strategy is *targeted trapping*, in which the vector also contains regions homologous to the targeted gene, thereby facilitating the deletion of a specific gene.[16,63]

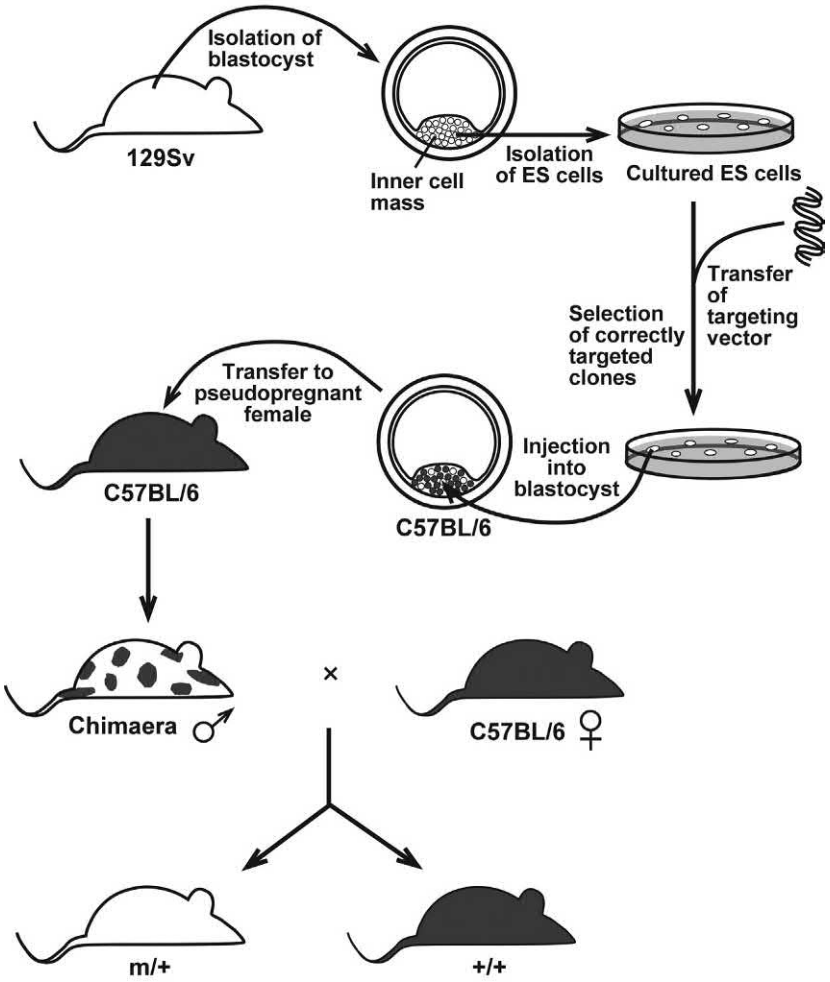


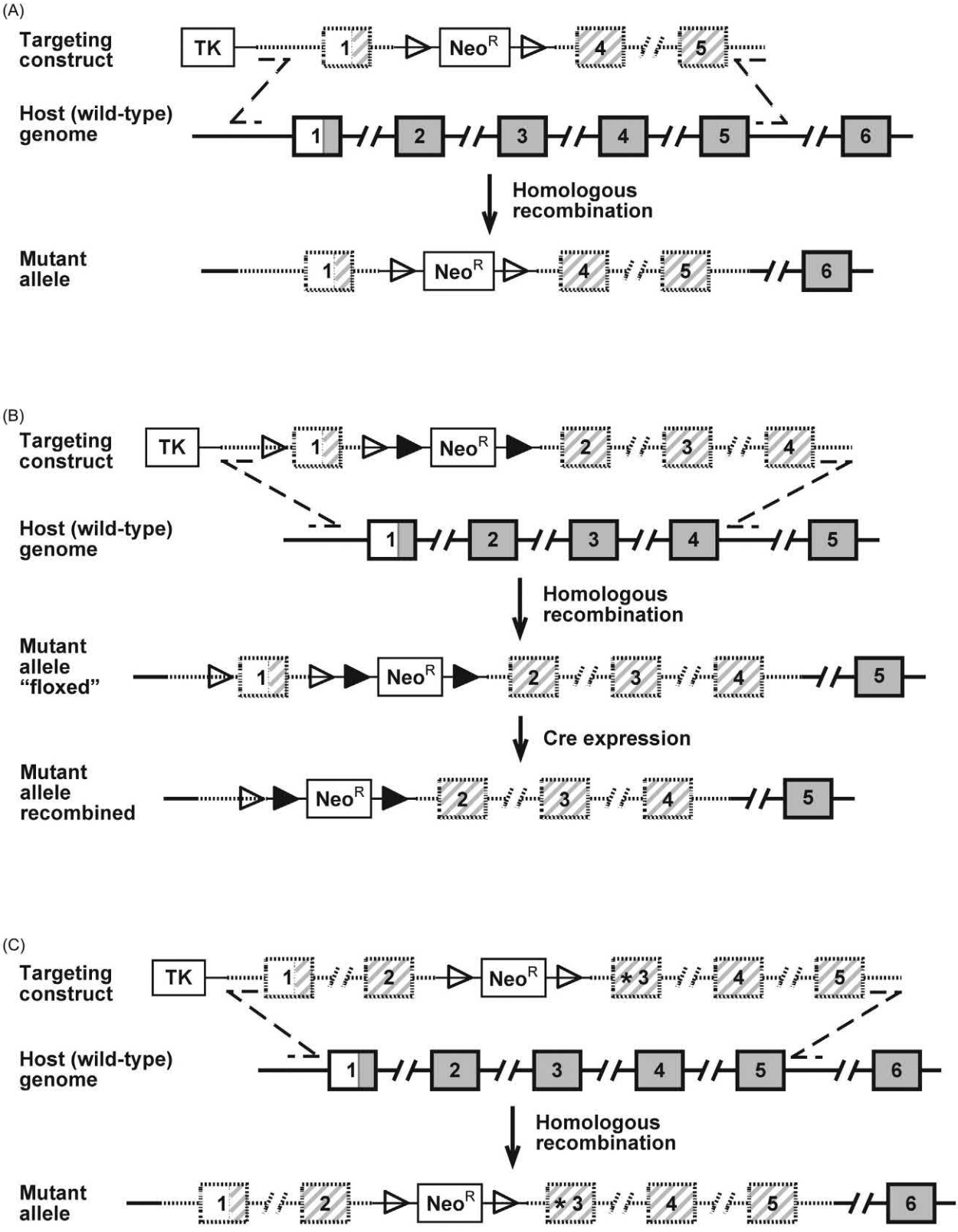
FIGURE 7.2 Gene targeting by modification of embryonic stem (ES) cells. Totipotent ES cells are isolated from the inner cell mass of a blastocyst [e.g., from a 129Sv embryo (shown)] and cultured. The targeting vector is transferred to the ES cells, and those in which homologous recombination or integration has been successful are selected. These are injected into the inner cell mass of a blastocyst from a different mouse strain [e.g., C57BL/6 (shown)], which is transferred to the uterus of a pseudopregnant female. The resulting chimeric offspring (usually males are selected) are bred with wild type, for example, C57BL/6 mice (usually females are selected) to achieve germline transmission. m, Mutant allele; +, wild-type allele. *Source: Reproduced from Piret SE, Thakker RV. Mouse models for inherited endocrine and metabolic disorders.* J Endocrinol *2011*;**211**:*211–30.*[1]

2.2 Targeted Knock-Out Strategies

A specific loss of function (i.e., knock out) of a gene of interest in the germline can be generated to yield *conventional targeted knock-out* models, as follows. A targeting construct is assembled, which contains two "arms" of sequence homologous to the gene of interest and that flank a positive selection cassette, such as the *Escherichia coli* neomycin phosphotransferase (Neo^R) gene (Fig. 7.3A). Integration of the Neo^R gene (and therefore the targeting construct) into the ES cell genome allows these ES cells to survive normally toxic amounts of antibiotic treatment, thereby allowing selection of ES cells that have been successfully targeted by homologous recombination. Furthermore, replacement of an exon (or exons) by the Neo^R cassette results in gene disruption, that is, "knock out"[17] (Fig. 7.3A). To further facilitate the selection of ES cells that have undergone successful targeting by homologous recombination, a negative selection cassette, such as the *Herpes Simplex* virus thymidine kinase (TK) gene, may also be used. The TK gene cassette is inserted at one end of the homologous region of the targeting construct, such that the TK cassette is lost if homologous recombination has occurred (Fig. 7.3A), but retained if nonhomologous recombination has occurred. In the presence of a thymidine analog in the growth medium, ES cells containing the TK cassette (i.e., following nonhomologous recombination) will not undergo cell division, as the thymidine analog will undergo phosphorylation and will be incorporated into the DNA by the TK, and thereby disrupt cell division, hence selecting out these ES cells. In contrast, those ES cells that do not have the TK cassette (i.e., following homologous recombination) will not have disrupted cell division due to incorporation of the thymidine analogue, and as a result will proliferate.[17] Correctly targeted ES cells are used to generate chimeric mice (Fig. 7.2), which are then bred with wild-type mice to yield mice with germline transmission of the disrupted allele, that is, "knock-out" mice, that have one copy of the disrupted allele in all of their cells. Cross-breeding of these heterozygous knock-out mice can then yield homozygous knock-out mice, which will have a disruption of both alleles of the gene in all of their cells. These "conventional" knock-out models have proved to be very useful in studies of human diseases, although their use may be limited if the disruption of the gene in a critical organ results in early death, for example, at any embryonic stage. To overcome such limitations, it may be useful to generate tissue-specific (i.e., *conditional knock out*) or time-specific (i.e., *inducible knock out*) models. This can be achieved by refining the gene-trap and "conventional" knock-out strategies by the addition of either LoxP or flippase (FLP) recombinase target (FRT) sites in the targeting vector (Fig. 7.3B). LoxP and FRT sites are short-DNA sequences, which are recognized and acted upon by the enzymes Cre (*C*auses *re*combination) recombinase or FLP recombinase enzymes, respectively, and when inserted to flank the genomic region of interest, will result in either excision or inversion of the DNA flanked by the LoxP or FRT sequences, depending on whether the two sequences are in the same orientation (Fig. 7.3B), or opposite orientations, respectively. Thus, insertion of the LoxP and FRT sequences allows several variations on the knock-out mouse, including either tissue-specific (conditional) or time-specific (inducible) knock outs (Table 7.2). Thus, if mice containing alleles in which the exon containing the start codon is flanked by LoxP sites (floxed) or FRT sites (flirted) are crossed with transgenic mice expressing Cre or FLP under the control of tissue-specific promoters (e.g., the PTH gene promoter for parathyroid-specific expression), the gene of interest can be knocked out in a specific tissue (Fig. 7.3B). The inducible models utilize a fusion protein, such as a modified ligand-binding domain of the estrogen receptor fused to the Cre (Cre^{ER}) or FLP gene, which on administration of an estrogen receptor antagonist (tamoxifen), translocates to the nucleus to excise the floxed allele(s), thereby allowing the gene to be permanently knocked out at the desired time, which may be either during embryonic or neonatal development, or in adult life.[18] These conditional and inducible knock-out mouse models have proved particularly useful to overcome embryonic or early postnatal lethality, for example, in studies of Blomstrand's chondrodysplasia due to PTHR1 loss of function (Table 7.4), or to understand the role of a protein in one particular tissue.

2.3 Targeted Knock-In Strategies

Knock-out mice have been very valuable for the study of physiological functions of proteins and the elucidation of disease mechanisms. However, knock-out models are not always the most appropriate, particularly when the human disease being studied is not due to a loss-of-function or null allele for the gene. Indeed, the majority of human diseases are unlikely to be due to null alleles, but are instead associated with point mutations, which may result in a constitutively active protein, or a toxic gain of function, as illustrated by PTHR1 mutations in Jansen's disease (Table 7.4), or dominant negative effects. Thus, to generate appropriate murine models for these diseases, the specific mutation needs to be introduced into the mouse genome, and this may be achieved by targeted knock-in or transgenic approaches (Tables 7.1 and 7.2). The generation of *targeted knock-in* models utilizes a similar approach to that described earlier for targeted knock-out models, except that a targeting vector which carries the desired mutation must be specifically generated (Fig. 7.3C). In addition, the positive selection cassette is normally placed in an intron and floxed so that it can be excised and cause minimal effects on gene expression.[19] The generation of *transgenic* models utilizes a targeting construct that usually contains the cDNA carrying the mutation, together with an appropriate promoter and poly(A) sequence, which is injected into the pronucleus


(A)
Targeting construct
TK
1
Neo^R
4
5
Host (wild-type) genome
1
2
3
4
5
6
Homologous recombination
Mutant allele
1
Neo^R
4
5
6
(B)
Targeting construct
TK
1
Neo^R
2
3
4
Host (wild-type) genome
1
2
3
4
5
Homologous recombination
Mutant allele "floxed"
1
Neo^R
2
3
4
5
Cre expression
Mutant allele recombined
Neo^R
2
3
4
5
(C)
Targeting construct
TK
1
2
Neo^R
*3
4
5
Host (wild-type) genome
1
2
3
4
5
6
Homologous recombination
Mutant allele
1
2
Neo^R
*3
4
5
6

of fertilized mouse eggs.[20,21] The transgene undergoes random insertion into the genome, and several copies are often inserted together, which therefore generates an overexpression model. Newly developed techniques for generating knock-in mouse models utilize genome editing. The general approach relies on the ability to target nucleases to specific DNA sequences. Thus, rather than relying on low-frequency spontaneous double-strand breaks followed by repair, as in the targeted knock-out and knock-in strategies described earlier, DNA strand breaks are induced at specific locations, followed by either nonhomologous repair or homologous repair if a template is also added (Fig. 7.4). To date, two main systems have been successfully used in vivo and these are: transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short-palindromic repeats (CRISPR)-CRISPR–associated (Cas) proteins.[65,66] TALENs utilizes the DNA-binding domain of a TALE conjugated to an endonuclease, such as Fok1 (Fig. 7.4A). TALEs consist of 7–34 repeats of 33–35 amino acids, with each repeat able to bind to one DNA nucleotide. Amino acids 12 and 13 within each repeat, known as the repeat variable diresidue (RVD), determine which DNA nucleotide the repeat binds to, and since the nucleotide-RVD "code" is known, TALEs can be designed which bind to any nucleotide sequence.[65] Since Fok1 operates as a dimer, a pair of TALEs is required, which recognize adjacent target sites, in order to introduce double strand breaks (Fig. 7.4A). Thus, ES cells can be transfected with a plasmid encoding the designed TALEs conjugated to Fok1, along with a template for homologous repair if desired, and a mutation introduced.[65] By contrast, Cas proteins are themselves endonucleases, and require a separate guide RNA to target them to the DNA sequence of interest (Fig. 7.4B). Furthermore, a single Cas protein introduces double strand breaks, and hence only one Cas protein is needed for each target site. Thus, the CRISPR–Cas system has major advantages over TALENs, as the same Cas protein is used regardless of the target, and the specificity is achieved by small RNA molecules, rather than having to design new TALENs for each target site.[66,67] Modifications of CRISPR–Cas genome editing, such as gene silencing or activation have also recently been developed, making the CRISPR–Cas system a powerful tool for multiple genetic manipulations.[67] These newly developed genome editing techniques have been utilized to generate models for diseases, such as autoimmune disorders and immune deficiencies, but not yet for bone and mineral disorders. As reviewed later, these different strategies for generating mouse models of human diseases have greatly facilitated studies of inherited bone and mineral disorders that have investigated mechanisms and treatments, which would not be easily feasible in patients.

3 GENETIC BONE DISEASES ASSOCIATED WITH DEFECTIVE CALCIUM HOMEOSTASIS

Heritable disorders of calcium homeostasis are most often caused by altered parathyroid gland function or PTH signaling, and/or altered renal calcium handling. These abnormalities of calcium homeostasis may be associated with hypocalcemia (Tables 7.3–7.5) or hypercalcemia (Tables 7.4 and 7.5), as well as skeletal dysplasias and disorders of bone undermineralization (Tables 7.3–7.5). The studies of some mouse models for disorders of parathyroid gland development, PTH signaling and the CaSR are reviewed as they provide examples for the variety of methods that have been utilized for generating different types of models, as well as important advances in our understanding of the genetic and physiological mechanisms of calcium homeostasis.

3.1 Disorders of Parathyroid Development

Defects in parathyroid gland development result in primary hypoparathyroidism, which may occur as an idiopathic endocrinopathy, or as part of a syndrome, such as the DiGeorge syndrome (DGS). Forms of familial isolated hypoparathyroidism (FIH) with autosomal dominant, autosomal recessive, and X-linked recessive

◀ **FIGURE 7.3 Strategies for targeted mutagenesis in ES cells.** Targeting vectors (dotted lines/striped boxes) typically consist of two "arms" of sequence homologous to the target gene flanking a positive selection cassette, such as the neomycin phosphotranserase (Neo^R) gene, and with a negative selection cassette, such as the thymidine kinase (TK) gene at one end of the construct. The Neo^R cassette is usually flanked by two LoxP sites (open triangles) so that it may be removed by expression of Cre recombinase after homologous recombination (dashed lines) with the host (wild-type) genome (solid lines). When homologous recombination occurs, the negative selection cassette is lost. Thick lines denote sequences derived from genomic DNA, with filled/striped boxes representing coding exons and open boxes representing noncoding exons; thin lines denote sequences derived from vectors. (A) Conventional knock out. In a typical conventional knock-out targeting vector, the Neo^R cassette replaces one or more exons, and is then excised by Cre recombinase after homologous recombination. (B) Conditional or inducible knock out. The Neo^R cassette may be flanked by flippase (FLP) recombinase target (FRT) sites (filled triangles) to allow removal by expression of FLP. Part of the coding region of the gene is also flanked by LoxP sites (open triangles). Thus, when homozygote mutant mice are crossed with mice expressing Cre in a tissue-specific manner, or mice expressing an inducible Cre, the gene is knocked-out within the tissue or upon administration of the inducer. (C) Knock in. A specific mutation is introduced into the targeting vector (*asterisk*), usually by site-directed mutagenesis. The Neo^R cassette is placed in an intron close to the mutation and excised after homologous recombination, either by introduction of Cre recombinase into the ES cells, or by breeding mutant mice with mice with ubiquitous Cre expression. *Source: Adapted from Piret SE, Thakker RV. Mouse models for inherited endocrine and metabolic disorders.* J Endocrinol *2011*;**211**:*211–30.*[1]

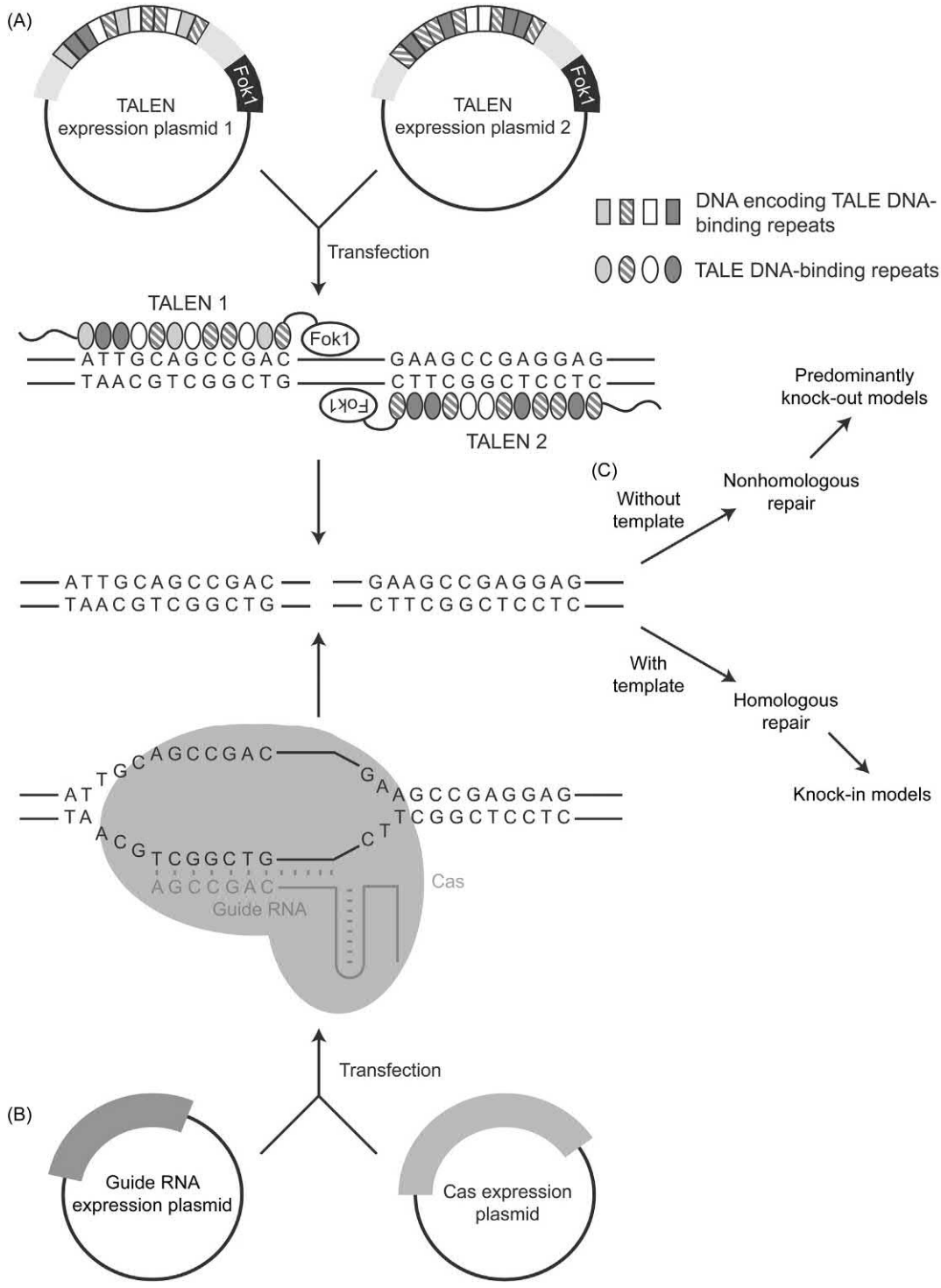


FIGURE 7.4 Gene editing using TALENs and CRISPR–Cas. (A) TALENs require transfection of two expression plasmids encoding two sequence-specific TALEs designed to bind to the DNA region of interest, conjugated to an endonuclease, for example, Fok1. The sequence specific TALEs bind to the sequences of interest, and bring together the two Fok1 monomers, which combine to introduce a double-strand break into the DNA. (B) CRISPR–Cas requires transfection of two plasmids encoding a guide RNA and the Cas endonuclease. The guide RNA binds to the sequence of interest and recruits the Cas protein, which introduces a double strand break into the DNA. (C) If a repair template is not included, the double strand break is repaired by nonhomologous repair, which usually introduces insertions/deletions into the DNA, and predominantly results in knock-out models via frameshifts, deletions, and/or premature stop codons. If a repair template is included, homologous repair can be achieved, which may be used to introduce any mutation of interest and generate a knock-in model.

modes of inheritance have been described. Hypoparathyroidism is associated with hypocalcemia and hyperphosphatemia.

3.2 Models for the DiGeorge Syndrome Type 1 Due to TBX1 Mutations

DGS is an autosomal dominant disorder characterized by the occurrence of hypoparathyroidism, immunodeficiency, congenital heart defects, and deformities of the ear, nose, and mouth. It arises from a congenital failure in the development of the derivatives of the third and fourth pharyngeal pouches, with resulting absence or hypoplasia of the parathyroids and thymus. DGS was reported during the 1980s to be associated with unbalanced translocations and deletions involving chromosome 22q11.2, and this is referred to as DGS type 1 (DGS1). Further studies in 2003 identified mutations of the transcription factor, T-box 1 (TBX1) gene in patients with DGS1.[68,69] Mice that are deleted for *Tbx1* have developmental abnormalities of the pharyngeal arches (Table 7.3). Thus, heterozygous ($Tbx1^{+/-}$) mice have cardiac outflow tract abnormalities, which are defects of the fourth branchial pouch.[22–24] However, only homozygous knock-out ($Tbx1^{-/-}$) mice have all the developmental anomalies of DGS1 due to third and fourth branchial defects which include: thymic and parathyroid hypoplasia, abnormal facial structures and cleft palate, skeletal defects, and cardiac outflow tract abnormalities.[22–24] The basis of the phenotypic differences between DGS1 patients, who are heterozygous, and the $Tbx1^{+/-}$ mice remain to be elucidated. TBX1 dosage, together with downstream genes that are regulated by TBX1, could provide an explanation, but the roles of these putative genes in DGS1 remain to be elucidated. Several tissue-specific murine knock-out models of Tbx1 have also been generated (Table 7.3). Knock out of *Tbx1* in the pharyngeal endoderm led to neonatal death with malformations identical to $Tbx1^{-/-}$ mice, likely due to failure of pharyngeal pouch outgrowth.[26] Selective inactivation of Tbx1 in the otic vesicle led to an absence of the inner ear.[27] Mesoderm-specific knock out of *Tbx1* led to multiple phenotypes that included malformation of the inner ear, defective pharyngeal patterning, cardiovascular defects, defective development of the proximal mandible, thyroid hypoplasia, and delayed ossification of the cranial bones.[25,28–31] An "allelic series" of Tbx1 expression showed tissue-specific dosage effects, demonstrating that cardiac outflow tract development is more susceptible to loss of TBX1 than craniofacial development.[70] Recent studies have shown that TBX1 may have a direct role in bone. Thus, mice with osteoprogenitor-specific knock out of *Tbx1* exhibited reduced ossification of the hyoid bone, significantly smaller parietal bones, and hypoplastic clavicles.[25] This was likely due to impairment of osteoblast differentiation, as the expression of markers of osteoblast differentiation, such as RUNX2 and bone sialoprotein was decreased in bones of $Tbx1^{-/-}$ mice; indeed TBX1 was shown to directly regulate RUNX2 expression.[25] These different mouse models have helped to elucidate the roles of TBX1 in organogenesis and DGS, and some of the transcriptional cascades required for development of these structures.

3.3 Models for Familial Isolated Hypoparathyroidism Due to PTH and GCMB Mutations

Both autosomal dominant and autosomal recessive forms of FIH have been found to be due to mutations in PTH itself and glial cell missing homolog B (GCMB), which is a member of a small family of GCM transcription factors whose expression appears to be restricted to the developing and adult parathyroid glands in mice.[71] FIH-associated PTH and GCMB mutations likely lead to a loss of function, and knock-out mice for Pth and the mouse GCMB homologue Gcm2, develop hypoparathyroidism (Table 7.3). *Pth* heterozygous ($Pth^{+/-}$) mice were viable with no apparent phenotypic abnormalities.[32] However, PTH null ($Pth^{-/-}$) mice had enlarged parathyroid glands with an absence of PTH expression, but with substantial CaSR expression. $Pth^{-/-}$ mice also had abnormal skull formation with enhanced mineralization, shortening of the long bones and other skeletal abnormalities.[33] When maintained on a normal calcium diet, $Pth^{-/-}$ mice developed hypocalcemia and hyperphosphatemia, consistent with hypoparathyroidism, that was associated with an decreased serum $1,25(OH)_2D_3$ concentration.[32] However, when given a low-calcium diet, serum $1,25(OH)_2D_3$ concentrations increased in the $Pth^{-/-}$ mice, and this led to increased bone resorption and stabilization of the low serum calcium at the expense of osteopenia.[32]

Mice lacking a copy of Gcm2 ($Gcm2^{+/-}$) appear normal, viable, and fertile. However, $Gcm2^{-/-}$ mice have no parathyroid glands, resulting in hypocalcemia and hyperphosphatemia, consistent with hypoparathyroidism. $Gcm2^{-/-}$ mice also have a mild bone phenotype, consisting of a low number of osteoclasts and osteoblasts, and increased bone volume. Yet, in contrast to $Pth^{-/-}$ mice, PTH was detected in their serum, and this PTH was produced by a cluster of cells in the thymus.[10] A detailed analysis of $Gcm2^{-/-}$ embryos showed that a presumptive parathyroid domain was initially formed, with transient expression of CaSR, before being lost by embryonic day 12.5 (E12.5) due to apoptotic cell death of the parathyroid precursor cells.[72]

3.4 Disorders of PTH Signaling

Abnormalities of PTH signaling in target tissues may result in disorders that include several types of pseudohypoparathyroidism (PHP), Blomstrand's

chondrodysplasia, and Jansen's disease (see Chapters 35 and 36). Blomstrand's chondrodysplasia and Jansen's disease are due to loss-of-function or gain-of-function mutations in the type 1 PTH receptor (PTHR1), respectively, while PHP is due to loss-of-function mutations in a G-protein ($G_s\alpha$) associated with the downstream signaling pathways of PTHR1.

3.5 Blomstrand's Chondrodysplasia

Blomstrand's chondrodysplasia is an autosomal recessive disorder, characterized by accelerated chondrocyte differentiation, dramatically advanced skeletal maturation, high bone density, short and poorly modeled long bones, and early death.[73] Blomstrand's chondrodysplasia is caused by homozygous loss-of-function mutations in PTHR1, resulting in a loss of PTH signaling. As PTHR1 also responds to PTH-related protein (PTHrP), loss of PTHrP signaling accounts for the additional features of abnormal breast development and tooth impaction in Blomstrand's chondrodysplasia.[74] Most homozygous PTHR1 knock-out (*Pthr1*$^{-/-}$) mice typically died in midgestation due to abrupt cardiomyocyte death, possibly as a result of defective intracellular calcium ion (Ca^{2+}) homeostasis.[34,35] Analysis of E18.5 embryos showed abnormalities throughout the skeleton, including reduced skeletal size, skull and mandible defects, excessive mineralization and lack of unmineralized cartilage, and shortened long bones.[34] Further analysis of embryos showed delayed chondrocyte differentiation at E15.5, and increased numbers of osteoblasts, increased cortical bone, and decreased trabecular bone at E18.5.[36] Generation of chimeric *Pthr1* knock-out mice containing different numbers of *Pthr1*$^{-/-}$ cells, in which the cells lacking *Pthr1* also contained the β-galactosidase gene, allowed the *Pthr1*$^{-/-}$ cells to be identified. This revealed abnormal ectopic differentiation of chondrocytes, and these differentiated *Pthr1*$^{-/-}$ chondrocytes were found to activate the Indian hedgehog (Ihh)/PTHrP signaling loop, which slowed differentiation of wild-type chondrocytes and led to defective mineralization of cartilaginous matrix, thereby demonstrating the importance of cell nonautonomous regulation of coordinated chondrocyte differentiation.[75] In contrast, chondrocyte-specific *Pthr1* knock out and *Pthr1* knock-down mice showed accelerated chondorocyte differentiation, thereby suggesting significant effects of loss of PTHR1 in other tissues on the conventional *Pthr1*$^{-/-}$ phenotype.[37] Postnatal ablation of *Pthr1* in chondrocytes in inducible chondrocyte–specific knock-out mice resulted in shortening of the long bones due to loss of growth plate chondrocytes, cartilage matrix and secondary ossification centers. This was associated with reduced proliferation and increased apoptosis of chondrocytes, thus indicating a requirement for PTHR1 signaling for the survival of chondrocytes.[38] Postnatal ablation of *Pthr1* selectively in osteocytes in adult mice led to mild osteopenia, reductions in trabecular bone and bone mineral density (BMD). Interestingly, during dietary calcium restriction, these osteocyte-specific *Pthr1*$^{-/-}$ mice were not able to compensate by increasing PTH production, and developed hypocalcemia, raising the possibility that osteocytes may play a role in adult extracellular calcium homeostasis.[39] In contrast, a knock-in model expressing mutant PTHR1 which retained the ability to signal via adenylyl cyclase but specifically inhibited phospholipase C (PLC) signaling (i.e., a "partial" knock out), unlike *Pthr1* knock-out mice, had increased proliferation, but delayed differentiation, of chondrocytes, along with defective ossification.[76] This phenotype was similar to overexpression of PTHR1, thereby indicating that PLC signaling in chondrocytes may normally act to suppress PTHR1 signaling.[76]

3.6 Jansen's Disease

Jansen's disease is an autosomal dominant disorder characterized by hypercalcemia, hypophosphatemia, normal or undetectable serum PTH levels, and short-limbed dwarfism.[73] Jansen's disease is due to heterozygous activating mutations in PTHR1, which cause PTHR1 to remain active even in the absence of PTH. One such activating mutation is His223Arg, and transgenic mice expressing human PTHR1 with this 223Arg mutation in growth plates had reduced mineralization due to delayed endochondral bone formation, resulting in short and deformed hindlimbs. Chondrocytes showed increased proliferation, but markedly delayed maturation, that is, the opposite phenotype to chondrocyte-specific knock out of PTHR1.[41] Furthermore, when the mice expressing the Jansen transgene were crossed with *Pthr1*$^{-/-}$ mice, the cranial defects, early mineralization, shortened long bones, and abnormal chondrocyte differentiation of the *Pthr1*$^{-/-}$ mice were rescued.[77] Selective expression of the His223Arg transgene in osteoblasts in vivo caused an increase in both osteoblast and osteoclast numbers, resulting in increased trabecular bone volume and decreased cortical bone thickness;[42] and selective expression of the same transgene in osteocytes resulted in increased bone mass and bone remodeling due to increased numbers of osteoblasts and osteoclasts.[43] Many hormone receptors, including PTHR1, are desensitized by internalization. In vitro work showed that phosphorylation of PTHR1 is required for its internalization, and knock-in mice with mutant PTHR1 in which serine phosphorylation sites were changed to alanine residues, had normal plasma calcium with very-low plasma PTH levels, that is, an activation of PTHR1, thereby demonstrating the importance of desensitization by internalization of the PTHR1 in vivo.[11] When placed on a low-calcium diet, phosphorylation deficient PTHR1 mice also had reduced bone volume and trabecular number compared to wild-type mice.[12]

3.7 Pseudohypoparathyroidism

PHP is characterized by hypocalcemia and hyperphosphatemia; however, in contrast to primary hypoparathyroidism, serum PTH is raised, consistent with normal parathyroid gland function and instead peripheral PTH resistance. PHP occurring in association with Albright hereditary osteodystrophy (AHO), which is characterized by short stature, obesity, round face, subcutaneous and ectopic soft tissue ossification, brachydactyly, and other skeletal abnormalities, is referred to as PHP type Ia (PHPIa) (see Chapter 35). The occurrence of hypocalcemia and hyperphosphatemia, due to PTH resistance, in the absence of AHO is referred to as PHP type Ib (PHPIb), while the sole occurrence of AHO (i.e., without hypocalcemia and hyperphosphatemia) is referred to as pseuopseudohypoparathyroidism (PPHP).[78] PHPIa and PPHP are caused by heterozygous loss-of-function mutations in the *GNAS1* gene, which encodes the alpha subunit of the G-protein Gs ($G_s\alpha$) that is coupled to PTHR1, and activates intracellular signaling in response to PTH or PTHrP stimulation. PHPIb is caused by disruption of imprinting control elements at the *GNAS1* locus, including intragenic deletion of exons of the syntaxin-16 (*STX16*) gene, and deletions of the whole neuroendocrine protein 55 (NESP55) differentially methylated region (DMR).[78]

PHPIa and PPHP may occur within the same family, and studies have shown that PPHP occurs in children of a father with PPHP or PHPIa, whereas children of a mother with PPHP or PHPIa develop PHPIa.[78] This is because of imprinting of the *GNAS1* gene. Thus, PHPIa is only manifested if the mutated allele is inherited from the mother, since *GNAS1* is predominantly expressed from the maternal allele in several endocrine tissues, that is, *GNAS1* is an imprinted gene.[78] Studies of heterozygous mice lacking *Gnas1* have further revealed the basis for PHPIa and PPHP. Heterozygous knock-out mice lacking exon 1 or exon 2 of *Gnas1* have decreased postnatal survival. However, PTH resistance was seen in surviving mice with a maternally inherited null allele (−m/+), but not mice with a paternally inherited null allele (+/−p).[45,46] $G_s\alpha$ expression was reduced in −m/+ mice compared to +/−p mice in some tissues, for example, renal cortex and thyroid, but not in other tissues, for example, renal medulla and heart, demonstrating tissue-specific imprinting of *Gnas1*.[45,46] Exon 1 heterozygous mice developed subcutaneous ossifications, starting with rare lesions at 3 months, and affecting all mice by the age of 12 months.[47] However, fewer and less severe lesions were detected in females than in males, regardless of maternal or paternal inheritance of the null allele, suggesting that ossification is a consequence of haploinsufficiency of $G_s\alpha$ and gender, rather than parental imprinting. These ossifications consisted of mineralized bone, which expressed osteoblast markers, such as osteopontin and osteonectin.[47] Heterozygous mice lacking a copy of the putative imprinting control region (ICR) for *Gnas1* were generated, and when mated with mice carrying an ENU-induced mutation in exon 6 of *Gnas1*, double heterozygotes in which the exon 6 mutant *Gnas1* allele was maternally inherited, had a partially restored response to PTH due to restoration of expression of $G_s\alpha$ from the paternal allele, demonstrating the importance of the ICR for *Gnas1* expression.[50] Chondrocyte-specific $G_s\alpha$ knock-out mice had short limbs, domed skulls, and epiphyseal and growth plate defects. The tibial epiphyses were shortened due to a reduced number of proliferating chondrocytes, which also had accelerated hypertrophic differentiation, similar to the chondrocyte-specific PTHR1 knock-out mice.[48] In addition, tibiae developed ectopic cartilage in the metaphyseal region, which contained mature chondrocytes.[48] In osteoblast/osteocyte-specific $G_s\alpha$ knock-out mice, early bone development (pre-E14.5) was normal; however, these mice had shortening of the long bones, and defects in bone formation, with reduced trabecular bone volume due to defective osteoblast maturation.[49] In contrast, cortical bone was thickened, likely due to reduced osteoclasts resulting in decreased bone resorption. Newborn mice also had craniofacial abnormalities due to premature ossification of cartilage.[49] Ablation of $G_s\alpha$ earlier in the osteoblast lineage substantially reduced bone formation, causing severe osteoporosis and multiple fractures at birth, likely due to premature maturation of osteoblasts to osteocytes, thus depleting osteoblast numbers.[79]

Several unrelated families with PHPIb have a 3 kb deletion which encompasses exons 4–6 of the *STX16* gene.[52] However, mice with deletion of exons 4–6 of *Stx16* had normal serum calcium and PTH concentrations regardless of maternal or paternal inheritance, and no epigenetic abnormalities were detected when compared to wild-type mice.[52] In contrast, targeted deletion of the *Nesp55* DMR resulted in hypocalcemia, hyperphosphatemia, and secondary hyperparathyroidism when inherited on the maternal allele but not when inherited on the paternal allele, thereby generating a model for PHPIb.[53] These *Gnas* mouse models are thus important tools to understand both the mechanisms of normal and ectopic ossification, and the regulation of gene expression by parental imprinting.

3.8 Disorders of the Calcium-Sensing Receptor (CaSR)

The CaSR is a 1078 amino acid G-protein coupled receptor with 7 transmembrane domains and a large 612 amino acid extracellular domain, and is predominantly expressed in the parathyroid glands and kidneys.[80,81] The CaSR is pivotal in extracellular calcium homeostasis

by mediating alterations in the release of PTH from the parathyroid glands in response to changes in extracellular Ca^{2+} concentrations.[80,81] CaSR mutations resulting in loss-of-function are associated with two hypercalcemic disorders, which are familial benign hypercalcemia, also referred to as familial hypocalciuric hypercalcemia (FHH), and neonatal severe hyperparathyroidism (NSHPT)[82]; while gain-of-function CaSR mutations result in two hypocalcemic disorders, which are autosomal dominant hypocalcemia with hypercalciuria (ADHH),[83,84] and a form of the Bartter syndrome.[85,86]

3.9 FHH and NSHPT Due to Loss-of-Function CaSR Mutations

FHH is an autosomal dominant disorder caused by heterozygous loss-of-function CaSR mutations. NSHPT is a life-threatening disorder characterized by severe neonatal hypercalcemia and undermineralization of bones and multiple fractures, which may be caused by homozygous, compound heterozygous, or de novo or paternally transmitted heterozygous loss-of-function CaSR mutations.[80,81] These loss-of-function CaSR mutations consist of nonsense mutations, frameshifting insertions and deletions, and missense mutations that result in an alteration of the CaSR such that the extracellular calcium concentration at which the defective CaSR produces a half-maximal response (EC_{50}) is significantly raised. To provide models for FHH and NSHPT, *Casr* knock-out mice were therefore generated[54] (Table 7.4). *Casr* heterozygous knock-out ($Casr^{+/-}$) mice had modest hypercalcemia with relative hypocalciuria and inappropriately elevated serum PTH, consistent with the features observed in FHH patients[54] (Table 7.4). $Casr^{-/-}$ homozygote knock-out mice had early onset of severe hypercalcemia in association with increased serum PTH concentrations, parathyroid hyperplasia, and bone demineralization. Moreover, $Casr^{-/-}$ mice died between 3 and 30 days after birth.[54–56] These findings in the $Casr^{-/-}$ mice are representative of the features found in patients with NSHPT.[80]

Further insights into the physiological role of the CaSR in extracellular calcium homeostasis have been gained by breeding *Casr* knock-out mice with *Pth*, *Gcm2*, and *Cyp27b1* (vitamin D 1-α-hydroxylase) knock-out mice. Thus, $Casr^{-/-}$ $Pth^{-/-}$ mice did not have increased neonatal lethality or skeletal abnormalities.[87] Furthermore, the overall mean values for serum Ca^{2+} concentrations and urinary Ca^{2+} excretion were similar in $Casr^{-/-}$ $Pth^{-/-}$ mice and wild-type control mice; however the fine control of calcium homeostasis was lost in the $Casr^{-/-}$ $Pth^{-/-}$ mice, which had a much larger range of serum Ca^{2+} concentrations. These findings demonstrate that the CaSR has PTH-independent roles in calcium homeostasis, since normal Ca^{2+} excretion did not require PTH.[87] Furthermore, a hypercalcemic challenge, induced by increased oral calcium intake, PTH infusion, or dietary phosphate deficiency, resulted in a greater elevation of serum Ca^{2+} in the $Casr^{-/-}$ $Pth^{-/-}$ mice, which had a reduced renal calcium clearance and failed to increase serum calcitonin, when compared to $Casr^{+/+}$ $Pth^{-/-}$ and $Casr^{+/-}$ $Pth^{-/-}$ mice.[88] However, infusion of PTH in $Casr^{-/-}$ $Pth^{-/-}$ mice further reduced the renal calcium clearance, thereby demonstrating that PTH can increase the CaSR-independent Ca^{2+} reabsorption by the kidney. Thus, it appears that PTH is necessary for the maintenance of normal serum calcium concentrations when facing a hypocalcemic challenge, but that PTH-independent mechanisms, such as CaSR-stimulated CT-secretion and increased renal Ca^{2+} excretion, are involved in maintaining normal serum calcium concentrations in response to hypercalcemic challenges.[88]

$Casr^{-/-}$ $Gcm2^{-/-}$ mice, which lack parathyroid glands, also do not have the increased neonatal lethality, skeletal abnormalities, or hypercalcemia observed in $Casr^{-/-}$ $Gcm2^{+/+}$ mice.[89] Interestingly, $Casr^{-/-}$ $Gcm2^{-/-}$ mice did not appear to demonstrate the large variability in serum Ca^{2+} levels that was observed in $Casr^{-/-}$ $Pth^{-/-}$ mice, and this may be due to the residual PTH production by the thymus.

$Casr^{-/-}$ $Cyp27b1^{-/-}$ mice had improved survival rates and body size compared to $Casr^{-/-}$ mice, and normocalcemia. However, $Casr^{-/-}$ $Cyp27b1^{-/-}$ mice had more severe hypophosphatemia and more marked PTH elevations, in association with significant parathyroid hyperplasia, compared to $Casr^{-/-}$ mice, indicating a role for $1,25(OH)_2D$ in suppression of PTH secretion.[90] The reduced bone mineralization detected in $Casr^{-/-}$ mice was partially ameliorated in $Casr^{-/-}$ $Cyp27b1^{-/-}$ mice; however, the $Casr^{-/-}$ $Cyp27b1^{-/-}$ mice still had reduced bone mineralization compared to wild-type mice, likely due to the elevated PTH levels in the $Casr^{-/-}$ $Cyp27b1^{-/-}$ mice.[90]

The absence of bone abnormalities in either $Casr^{-/-}$ $Gcm2^{-/-}$ or $Casr^{-/-}$ $Pth^{-/-}$ mice, which respectively have no parathyroid glands or do not make PTH, suggested that the bone abnormalities in the $Casr^{-/-}$ $Gcm2^{+/+}$ and $Casr^{-/-}$ $Pth^{+/+}$ mice and in NSHPT patients are largely due to the primary hyperparathyroidism.[89] However, the expression of the CaSR in bone and cartilage also suggests a role for the CaSR in the skeleton and in contributing to the bone abnormalities observed in the $Casr^{-/-}$ $Gcm2^{+/+}$ and $Casr^{-/-}$ $Pth^{+/+}$ mice. An additional complicating factor is the presence of an alternative CaSR splice variant in the *Casr* knock-out mice, which were generated by insertion of the Neo^R cassette into exon 5 so as to disrupt the gene. However, this results in an incomplete knock out of the CaSR receptor because of alternate splicing of the *Casr* gene. The alternative spliced CaSR lacks the amino acid sequence encoded by exon 5, which encodes 77 amino acid residues of the extracellular

domain. This spliced receptor lacking the protein from exon 5 ($^{Exon5(-)}$Casr) is shorter, but retains the majority of the extracellular domain, all of the seven transmembrane domains and the cytoplasmic domain, and is able to compensate for the absence of full length CaSRs in bone and cartilage.[91] Thus, the $^{Exon5(-)}$*Casr* knock out is incomplete.[91] In order to explore further the skeletal role of the CaSR, tissue-specific knock outs of the *Casr* which lacked exon 7 (that encodes the 501 amino acids which form the 7 transmembrane domains and 4 intracellular loops) were generated in the parathyroid glands, bone, and cartilage (Table 7.4). Parathyroid gland–specific homozygous *Casr* knock-out (PT-KO) mice died ~2 weeks after birth and were much smaller than wild types. The *Casr* PT-KO mice had severe marked hypercalcemia with elevated serum PTH concentrations, and increased urinary Ca^{2+} excretion, which is different compared to the conventional $Casr^{-/-}$ mice, due to the presence of functional CaSR in the kidney.[57] *Casr* PT-KO mice also had severe undermineralization of the skeleton, consistent with NSHPT. Heterozygous (PT-Het) mice had mild hyperparathyroidism.[57] Selective knock out of the *Casr* in osteoblasts by utilizing a Collagen I-Cre to generate heterozygous (COL-Het) and homozygous (COL-KO) knock-out mice, revealed COL-KO mice to have a similar skeletal phenotype to the PT-KO mice, with multiple factures and profound undermineralization, due to a lack of differentiation, and increased apoptosis, of osteoblasts.[57] Homozygous knock out of the *Casr* in growth plate cells caused embryonic lethality at ~E12–E13. To overcome this, an inducible growth plate–specific knock out was generated via the use of an ER-Cre. The *Casr* was electively knocked out in growth plates before birth, and this also resulted in bone defects, such as short bones, as well as a decrease in mature and terminally differentiated chondrocytes.[57] Further evidence for the role of CaSR in bone responses to PTH comes from studies in which $Pth^{-/-}$ mice and $Casr^{-/-}$ $Pth^{-/-}$ mice were infused with PTH. Continuous infusion of PTH into $Pth^{-/-}$ mice resulted in the expected increases in cortical bone turnover and decreases in cortical bone volume, bone mineral content and BMD, but these responses were blunted in $Casr^{-/-}$ $Pth^{-/-}$ mice, indicating an important role for the CaSR in modulating the effects of PTH on cortical bone.[92] Thus, these tissue-specific and double knock-out models demonstrate a role for the CaSR in bone and calcium homeostasis, and indicated that the skeletal abnormalities seen in patients with NSHPT may not be solely due to the primary hyperparathyroidism.

3.10 ADHH Due to Gain-of-Function CaSR Mutations

Gain-of-function CaSR mutations are associated with the disorder of autosomal dominant hypocalcemia type 1 (ADH1). The hypocalcemia is usually mild and asymptomatic, but may sometimes be associated with tetany and seizures. Hyperphosphatemia and hypomagnesemia are also notable features, and the circulating PTH concentrations are usually in the low–normal range.[83] The CaSR mutations resulting in ADH1 are invariably heterozygous missense mutations that result in a lower EC_{50}. To generate a mouse model for ADH1 and such an activating CaSR mutation, two possible strategies could have been utilized, which were a knock-in approach or searching for a chemical induced mutation (Table 7.2, Figs. 7.1 and 7.3). Indeed, in 2004, a search of mice derived from use of the chemical iPMS to produce mutants, identified a mouse model for ADH1 (Table 7.4). This mouse model, which was first noted to feature opaque flecks of the lens and designated *Nuf* (nuclear flecks),[9] has hypocalcemia and hyperphosphatemia in association with inappropriately low plasma PTH concentrations. In addition, mutant mice may also have widespread ectopic calcification and suffer from sudden death.[9] These phenotypic features in the mutant *Nuf* mice are due to a CaSR missense mutation, Leu723Gln, which results in a reduced EC_{50}, consistent with an activating CaSR mutation.[9] *Nuf* mice have also been used in preclinical studies of a CaSR negative allosteric modulator (known as a calcilytic), NPS 2143. When administered by i.p. injection, NPS 2143 elicited a rise in plasma Ca^{2+} and PTH in both heterozygous and homozygous *Nuf* mice.[93] Two knock-in models for ADH1 have recently been generated, in which the endogenous mouse *Casr* gene was replaced with the human *CaSR* gene harboring either the Cys129Ser or Ala843Glu ADH1 mutations. Both of these models developed hypocalcemia and hyperphosphatemia with inappropriately low plasma PTH levels, and were also hypomagnesemic and hypercalciuric with reduced plasma $1,25(OH)_2D_3$ levels, and when fed a high-Ca^{2+} diet to correct the hypocalcemia, developed renal calcification.[58] Treatment of both Cys129Ser and Ala843Glu knock-in mice with the calcilytic JTT-305/MK-5442 resulted in increased plasma Ca^{2+} and PTH levels, reduced plasma phosphate levels, and reduced urinary Ca^{2+} excretion, in contrast to treatment with PTH(1-34), which did not suppress urinary Ca^{2+} excretion.[58] Thus, the *Nuf* and Cys129Ser and Ala843Glu knock-in mouse models for ADH1 due to activating mutations of the CaSR have been utilized for preclinical studies, and may also be an important tool to understand the mechanisms of ectopic calcification and the contribution of the CaSR to renal Ca^{2+} handling.

4 CONCLUSIONS

Mouse models for inherited disorders of mineral and skeletal homeostasis have been generated by a variety of strategies, and investigations of these have provided

new and valuable insights into the pathogenesis of these often-complex conditions. These murine models have also helped to increase our understanding of the relationships between different signaling components involved in skeletal biology, and calcium and phosphate homeostasis. Mouse models as preclinical models also provide opportunities for assessing future novel therapies for bone and skeletal disorders.

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CHAPTER

8

Prospects of Gene Therapy for Skeletal Diseases

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1 INTRODUCTION

Gene therapy refers to the strategy by which nucleic acids, such as genes, microRNAs (miRNAs) and short-interfering RNAs (siRNAs), are delivered to target tissues using naked oligonucleotides, viral vectors or nonviral vectors to prevent, treat, or cure disease. In its infancy, the gene therapy field focused on use of viral vectors to deliver genes encoding wild-type proteins for the purposes of replacing absent or aberrant proteins produced due to genetic mutations. However, as time has progressed, gene therapy has been adopted to address more complex disease states, including nonhealing fractures (termed "nonunion fractures") and arthritis, where pathogenesis is typically not caused by a single mutation but by complex interactions between genetics and the environment. In this regard, the approach is typically used to introduce factors to overcome barriers to healing, such as inflammation, while enhancing tissue regeneration.

The foundations for gene therapy were first laid with the discovery that DNA encoded genetic information and could be transferred from cell to cell by conjugation, transformation, or transduction.[1] In the 1970s, following the discovery of the genetic code, Friedmann proposed gene therapy as a means with which to replace mutated genes and cure disease.[2] Almost 2 decades later, the first gene therapy trial was approved by the FDA and used white blood cells transduced ex vivo with the adenosine deaminase gene to treat two patients suffering from adenosine deaminase deficiency. Though a temporary response was observed in one patient, continued enzyme replacement therapy was ultimately required.[3] Despite this setback, modifications to viral vectors and transduction protocols would be met with success in future trials. Unfortunately, in 1999, progress and achievements celebrated by the field were overshadowed by the passing of Jesse Gelsinger—the first death directly related to a gene therapy vector.[4] This tragedy was soon followed by discovery of acute T-cell lymphoblastic leukemia or preleukemic myelodysplastic syndrome in patients with immune disorders, such as X-linked severe combined immunodeficiency, treated with gene therapies utilizing integrating viral vectors.[5,6] Together, these and other events highlighted the need for a deeper understanding of human immunology, and for improved vectors and approaches for gene therapy.

Despite the clinical setbacks seen in the 1990s and early 2000s, the field of gene therapy has celebrated some great successes in recent years. In particular, efficacious applications in human patients have been obtained in clinical trials for previously intractable inherited diseases, such as certain forms of immunodeficiency, neurologic disorders, blindness, hemoglobinopathy, and coagulation disorders.[7] Considering these successes and the number of ongoing clinical trials, there is renewed hope that gene therapy may still be a viable treatment option for monogenetic and multifactorial diseases.

In all cases, the success of any gene therapy is tightly linked to its ability to efficiently deliver genes to target cells and maintain gene expression over an extended period of time. As mentioned earlier, gene therapy vectors can be viral or nonviral in origin. Viruses have naturally evolved to efficiently transduce host cells, but safety issues surrounding their immunogenic profile and potential for integration mutagenesis remain a concern. While

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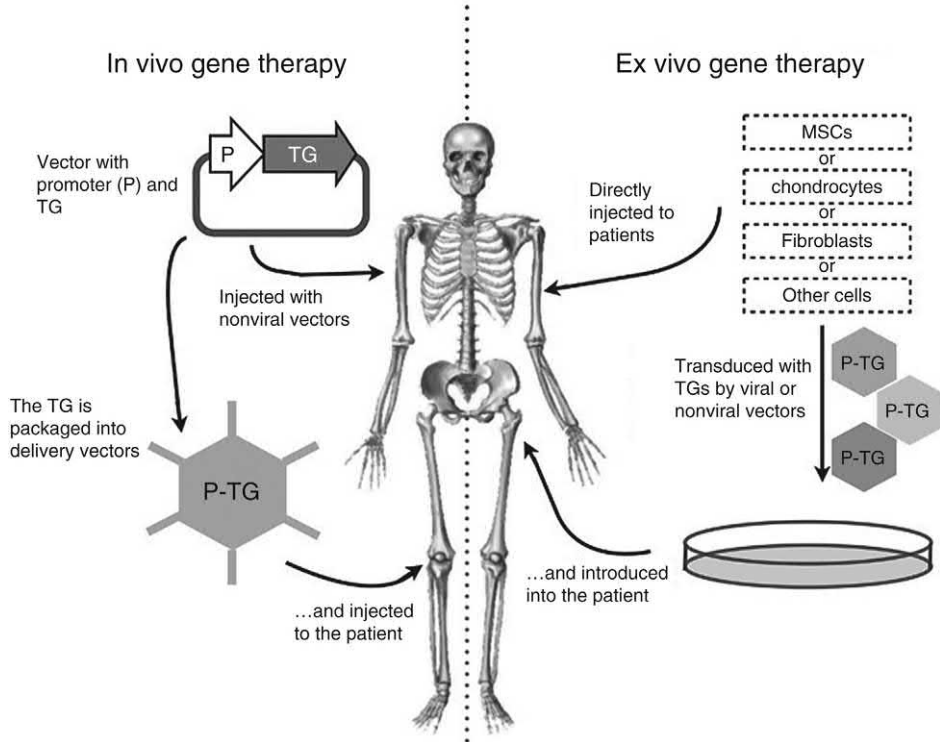

FIGURE 8.1 **An overview of gene therapy approaches.** *TG*, Therapeutic gene; *MSCs*, mesenchymal stem cells.

nonviral vectors are perceived to be a safer alternative, transduction efficiencies are far below what can be achieved with viral vector systems.

Gene therapies are designed and delivered using either an in vivo or ex vivo approach. The former involves administration of the vector directly into the organism, whereas the latter requires extraction of target cells, transduction with a vector and reintroduction of those manipulated cells into the organism. An overview of gene therapy approaches is illustrated in Fig. 8.1 and discussed below.

2 VECTORS IN SKELETAL GENE THERAPY

The choice of optimal vector is one key to successful gene therapy. Many vectors—viral and nonviral—exist, of which the most commonly used are discussed below. Key features of the various vectors are listed in Table 8.1.

2.1 Adenovirus

Adenoviruses (AdVs) belong to the family of Adenoviridae and consist of at least 55 immunologically distinct serotypes divided into 7 different species (A–G).[8,9] These nonenveloped viruses possess a linear double-stranded DNA (dsDNA) genome surrounded by a 100-nm icosahedral capsid formed of hexon and penton subunits with protruding fiber proteins that assist with binding of the virus to target cells.[10,11] For the widely used serotype 5 AdV (AdV5) vectors, the knob domain of the viral capsid's fiber protein binds to the coxsackievirus and adenovirus receptor (CAR) expressed on the target cell surface. This is followed by interactions with cell surface α_v integrins that lead to internalization via clathrin-mediated endocytosis.[12] Upon entry into the cytosol, the viral particle escapes the endosome and is disassembled, allowing the viral genome to translocate and replicate within the nucleus in an episomal state.[10,11]

The AdV genome is approximately 36 kb in size and consists of early (E1–E4) and late genes (L1–L5), which are transcribed before and after viral DNA replication, respectively.[11] To deliver therapeutic genes (TGs) using AdV vectors, the virus must be rendered replication-deficient by removal of genes required for its lytic life cycle. In first-generation AdV vectors, E1 was replaced with the TG of interest, and the E1 proteins required for virus production were provided in trans by a special cell line. The subsequent second-generation AdV vectors were generated by deletion of E2, E3, or E4 in various combinations. Despite absence of E1, the remaining viral genes are expressed at low levels in transduced cells, which is directly cytotoxic and can lead to long-term, chronic toxicity.[13] Further, expression of viral proteins can provoke an adaptive immune response against transduced cells, leading to loss of TG expression after short periods of time.[14]

TABLE 8.1 Commonly Used Gene Transfer Vectors and Their Key Features

Vectors	Particle size (nm)	Genome	Maximum transgene capacity (kb)	Transduced cell types
AdV	80–100	dsDNA	36	MSCs, synoviocytes, ligament cells, tendon cells
AAV	20	ssDNA	5	Chondrocytes, tendon cells, MSCs
RV/LV	80–100	ssRNA	12	MSCs, chondrocytes, periosteal cells
Naked DNA (biolistics, sonication, electroporation)	Depending on plasmid size	dsDNA	—	Muscle cells
Liposomes	Wide range	dsDNA	—	Perichondrial cells, chondrocytes, ligament cells, tendon cells
Nanoparticles (chitosan)	100–300	dsDNA	—	Chondrocytes

AAV, Adeno-associated virus; AdV, adenovirus; dsDNA, double-stranded DNA; LV, lentivirus; MSCs, mesenchymal stem cells; RV, retrovirus; ssDNA, single-stranded DNA; ssRNA, single-stranded RNA; —, unlimited size.

To overcome these issues, helper-dependent AdVs [also known as gutless, gutted, or high-capacity vectors (HDVs)] were developed, in which all viral coding sequences excluding the inverted terminal repeats and packaging signal are removed, and replaced with the TG of interest. To package the HDV for gene therapy, a helper virus is used to provide in trans all proteins necessary for viral particle assembly.[15] In addition to having a remarkable cloning capacity of up to 36 kb, the HDV vectors are safer and more efficacious compared to their first-generation predecessors. In fact, it has been shown that HDVs can mediate gene expression for up to 7 years in the liver of nonhuman primates.[16] Although HDVs were designed to minimize the host immune response, these too can activate an innate and adaptive immune response directed against the viral capsid or TG components.[17,18]

Overall, the features that make AdVs useful as gene therapy vehicles are their large genetic payload and their ability to efficiently infect dividing and quiescent cells in a wide range of tissues. Other advantages include the low risk for insertional mutagenesis due to infrequent integration into the host cell genome and relatively easy manipulation of the virus genome.

2.2 Adeno-Associated Virus

A member of the Parvoviridae family, adeno-associated virus (AAV) was discovered in 1965 as a contaminant in AdV preparations.[19] AAVs are naturally replication-deficient and require the assistance of a helper virus, such as AdV or herpes virus, to replicate. The virions are nonenveloped with an icosahedral capsid measuring about 20-nm in diameter surrounding a linear, single-stranded DNA (ssDNA) genome.[11,20] Similar to AdVs, AAVs enter target cells through receptor-mediated endocytosis; however, variability in sequence identity within the capsid coding region leads to differences in tissue tropism amongst the serotypes.[21,22] Once in the endosome, the virus escapes into the cytosol where its single-stranded DNA genome is released and transported to the nucleus to be converted to dsDNA.[10,11]

The AAV genome is approximately 4.7 kb in size, and consists of 145-base inverted terminal repeats flanking two open reading frames (ORFs): the 5′-*rep* ORF (replication) and the 3′-*cap* ORF (capsid). Through alternative splicing, the 5′-*rep* ORF encodes for four distinct proteins essential for genome replication, encapsidation, and integration, whereas the three proteins that form the viral capsid are encoded by the 3′-*cap* ORF.[11,20] Though the AAV genome can specifically integrate into the human host cell genome at the AAVS1 locus on chromosome 19, the majority of AAV vectors exist in an episomal state.[23] Still, random integration can occur within genes as well as at regulatory, ribosomal, and palindromic DNA sequences.[24]

The major advantage of AAV is its low immunogenicity, which enables long-term gene expression in a variety of tissues.[25] AAV gene therapy vectors are generated by replacing the 5′-*Rep* and 3′-*Cap* genes with an expression cassette for the TG of interest. However, the utility of these vectors is limited to small transgenes owing to its small cloning capacity (maximum 4.7 kb). For TGs >4.7 kb in size, efforts have been made in recent years to increase cloning capacity of AAVs. In addition to limiting expression elements,[26] dual-vector systems have been developed in which the TG of interest is split between two distinct constructs and reconstituted by homologous recombination of overlapping sequences following transduction.[10] Unfortunately, this system yields relatively low expression when compared with a single vector approach. In addition to its limited cloning capacity, the single-stranded DNA genome of AAVs must be transformed into dsDNA before transcription can occur, resulting in delayed onset of gene expression.

This problem has been solved by packaging dsDNA into AAV capsids generating so-called self-complementary AAVs; however, the transgene capacity of these vectors is limited to approximately 2.5 kb.[27]

2.3 Retrovirus and Lentivirus

Members of the Retroviridae family can be divided into simple or complex viruses. Among the simple retroviruses [(RVs) oncogenic retroviruses], the murine leukemia virus is most commonly used as a vector in gene therapy, while the most commonly used vector among the complex RVs is lentivirus (LV).[28] Retroviridae are enveloped viruses with a diameter of 80–100-nm. The envelope is a lipid bilayer derived from the host cell membrane that also contains viral-encoded glycoproteins. The genome is a diploid (+) linear, single-stranded RNA (ssRNA) molecule approximately 7–12 kb in length. Simple RVs have a genome containing three major coding segments (*gag*, *pol*, and *env*) and one small coding domain (*pro*). The structural proteins of RVs are encoded by *gag*, whereas *pol* encodes enzymes including reverse transcriptase, integrase, and proteases that accompany the single-stranded RNA genome.[28,29] In addition to these genes, complex RVs carry six accessory genes responsible for regulating viral gene expression, replication, and packaging.[30]

In general, viral glycoproteins of RVs bind the cluster of differentiation 4 (CD4) receptor and deliver the viral core to the cytosol through fusion with the plasma membrane or receptor-mediated endocytosis.[31] Upon entry into the cell, viral reverse transcriptase converts the ssRNA genome into dsDNA. Since the DNA strand derived from simple RVs is not able to pass through the nuclear membrane, integration can only occur in mitotically active cells. In contrast, the LV genome encodes the viral matrix protein gag and accessory viral protein vpr that permit nuclear entry of viral nucleic acids in the absence of mitosis;[28,32] as such, LVs can be used to infect both dividing and nondividing cells. Following reverse transcription, the DNA strand of the RV is integrated into the host cell genome through interactions between long terminal repeat-localized integrase attachment sites and the viral integrase.

For gene therapy purposes, all viral genes of the RV genome are deleted to render the vectors replication-deficient and provide space for insertion of the TG of interest. Compared to AdV and AAV, RV-based vectors show relatively low transduction efficiency and restricted tropism to CD4(+) cells in vivo. As such, investigation has focused on altering the retroviral capsid proteins (termed pseudotyping) to increase the number of cell types susceptible to transduction. For instance, the vesicular stomatitis virus glycoprotein has been used to target cells expressing low density lipoprotein receptor, glycoproteins from the measles virus have been employed to target lymphocytes and other immune cells,[33] and envelope proteins from Sendai virus permit transduction of epithelial cells.[34,35] Perhaps the most advantageous property of retroviral vectors is their ability to stably integrate and confer long-term transgene expression in rapidly dividing tissues. Further, use of tissue-specific promoters together with regulatory elements, such as the woodchuck hepatitis virus posttranscriptional regulatory element, have been employed to improve expression levels of the transgene while limiting promoter inactivation.[10] Though use of retroviral gene therapy has shown potential for treatment of human diseases, the risk of insertional mutagenesis is still a concern.

2.4 Nonviral Vectors

Apart from the viral vectors described earlier, a number of nonviral gene transfer mechanisms exist. Genes can be simply transferred into target cells as naked DNA; however, naked DNA has shown significant transfection efficiency only in skeletal muscle tissue, whereas in most other musculoskeletal tissues gene transfer was not sufficient. To increase transfection efficiency, a gene gun (biolistic particle delivery system), sonication, or electroporation can be used. While these methods are useful for in vitro gene transfer, their application in vivo is difficult except in the case of skeletal muscle and skin.

The transduction efficiency of naked DNA is generally low due to its negative charge and susceptibility to decay via actions of DNA degrading enzymes. To overcome these hurdles, DNA can be incorporated in or attached to liposomes or nanoparticles. Liposomes are spherical vesicles composed of a lipid bilayer that have been shown to transduce certain cell types in vitro and in vivo, while chitosan, a polysaccharide and naturally occurring biopolymer, is widely used to make biodegradable nanoparticles that encapsulate DNA. Transfection efficiency in vitro was further improved using hyaluronic acid/chitosan hybrid nanoparticles.[36]

2.5 Conclusions

Every vector type has its advantages and disadvantages. As such, a number of factors, including transduction efficiency, gene expression duration, transgene capacity, and method of delivery, need to be considered when choosing vectors for a particular therapeutic application. Furthermore, potential risks and ethical aspects with regard to integrating vectors need to be considered.

3 METHODS OF GENE DELIVERY

In addition to the selection of an appropriate viral vector, researchers must decide which method of delivery best suits their experimental or therapeutic needs.

In vivo gene therapy refers to direct administration of the viral vector into the organism, whereas ex vivo gene therapy requires extraction of cells from the patient, transduction with a vector and reintroduction of those manipulated cells back to the target site. Below the advantages and disadvantages of these two approaches are reviewed.

3.1 In Vivo Delivery

Of the two delivery strategies, in vivo approaches are simpler and less expensive. However, given that vectors are delivered directly into the systemic circulation or target tissue, immune responses to viral antigens are a significant clinical concern as they can limit transgene expression and produce a variety of adverse immunological reactions that, in severe cases, could result in death (Section 4). Additionally, as successful expression of TGs is predicated on the existence of a healthy cell population to be transduced, the lack of such cells in many disease or injury states can potentially limit this method's effectiveness.[37]

3.2 Ex Vivo Delivery

In a typical ex vivo approach, cells are isolated from either the patient (autologous) or a healthy donor (allogenic), expanded in culture, and transduced with the TG of interest before being implanted back into the patient. Though this method reduces exposure of the patient's immune system to viral antigens, a number of other concerns exist including rejection (in allogenic transfers), the need for multiple surgeries (in autologous transfers), gene transfer efficiency, stability of transgene expression, and the question of whether the genetically modified cells will engraft into the tissue.[38] Additionally, many primary cell types do not expand or transduce well in culture, making it difficult to obtain the quantity necessary for successful treatment.

Cell types commonly used in ex vivo approaches include mesenchymal stem cells (MSCs),[39] fibroblasts,[40] and native tissue cell types, such as chondrocytes.[41] With regards to usage of MSCs, it is the known that these cells release antiinflammatory and tissue remodeling factors at the site of delivery.[42] Though this could be beneficial in most instances of tissue injury or disease, lack of proper controls can make it difficult to determine what additional benefit the TG itself provides.

In addition to simply injecting transduced cells, many groups utilize natural or synthetic scaffolds—a concept borrowed from more traditional tissue engineering, in which cells are cultured onto the scaffolds and the entire scaffold/cell complex is added to the defect.[43] Use of gene-activated scaffolds, in which the vector is embedded within the matrix and transduces cells as they migrate in from the tissue, are also becoming more popular.[44]

4 THE IMMUNE RESPONSE TO GENE THERAPY VECTORS

The immune response to viral vectors (and even nonviral vectors) represents a significant challenge to the use of gene therapy in human patients. The immediate inflammation triggered by viral capsid proteins and nucleic acids is mediated largely by the innate immune system, whereas later phases of the immune response involve cellular and humoral aspects of the adaptive immune system. For simplicity, immune responses against AdV5 are described as an example given their prominence in gene therapy clinical trials.[45] For information on immune reactions to AAV, RV, and other AdV vectors, refer to the following excellent reviews.[46,47]

4.1 The Innate Immune Response

The innate immune response, which is responsible for the immediate recognition of and in vivo toxicity to viral vectors, can be divided into intrinsic and extrinsic components. The components of intrinsic innate immunity preexist within host cells and block viral replication directly,[48] whereas extrinsic mechanisms limit infection indirectly through receptor-mediated signaling and subsequent upregulation of chemokines and cytokines including interferons (IFNs) and interleukin-1 (IL-1).[49]

Before reaching the target cell, viruses are typically sequestered and destroyed by binding of various factors to the viral capsid, including clotting factors, immunoglobulins, and components of the complement system.[49,50] Once at the target cell, binding of the AdV capsid to CAR and α_v integrins initiates intracellular signaling that activates expression of nuclear factor-κB (NF-κB) target genes, such as IL-1 and tumor necrosis factor-α (TNF-α). Following internalization, the viral DNA is unpackaged and released into the endosomal lumen where it is recognized by Toll-like receptor 9, leading to upregulation of NF-κB-regulated chemokines and cytokines, and induction of type I IFNs.[49,50] Viral DNA that escapes the endosome and reaches the cytosol is detected by DNA-dependent activator of IFN-regulatory factors or nucleotide oligomerization domain-like receptors, which culminates in induction of an IFN or IL-1 response, respectively.[51,52] In general, IFNs, such as IFN-α, function in an autocrine and paracrine manner to inhibit viral replication and packaging, whereas IL-1 acts to upregulate chemokines and cytokines required for apoptotic induction of infected cells and recruitment of proinflammatory cell types.[49]

4.2 The Adaptive Immune Response

Within 3–7 days following viral infection, transduced cells process and present viral-derived peptides on major histocompatibility complex (MHC)-I to cytotoxic T-lymphocytes, which release factors that perforate the cell membrane to induce apoptosis. At the same time, virally transduced antigen presenting cells, such as dendritic cells, present viral proteins on MHC-II to CD4(+) and CD8(+) T-lymphocytes. These T-lymphocytes in turn activate B cells within lymph nodes, which differentiate into plasma cells and produce neutralizing antibodies against viral capsid antigens.[53]

One of the major limitations with viral vectors for gene therapy lies in the widespread global immunity to AdVs and AAVs. In addition to neutralizing the virus upon administration, neutralizing antibodies can activate complement and induce an inflammatory state.[54] The prevalence of neutralizing antibodies against either AdVs or AAVs varies depending on the serotype. For instance, 96% of individuals tested by Chirmule and coworkers were positive for antibodies against AdV capsid proteins; interestingly, serum from only 55% of these subjects was capable of neutralizing AdV5 infection in vitro .[55] In addition to AdVs, 35%–80% of individuals have humoral immunity for AAV2, whereas the less commonly used AAV7-9 exhibit only a 15%–30% seroprevalence.[46,56] Interestingly, seroprevalence to AAVs is also geographically dependent. For instance, more individuals in Africa and China have neutralizing antibodies to AAV1 compared to those in the USA.[57]

4.3 Strategies to Limit Immune Reactions to Gene Therapy Vectors

The various strategies used to circumvent the immune response to viral vectors have focused on altering the viral capsid to avoid an innate and/or humoral response or immune suppression to enhance transduction of target tissues.[46,53] With regards to the former, one approach has been to pseudotype viral vectors with capsid proteins from virions with little-to-no preexisting immunity in the human population. An alternative has been to disrupt specific epitopes within the viral capsid, but this is problematic owing to a lack of knowledge with regards to the characteristics of serotype-specific viral epitopes. To overcome this, error-prone PCR has been employed to generate capsid variant libraries that can be screened for resistance to serum neutralization.[58] Conjugation of the vector with polyethylene glycol (PEG), a synthetic polymer that exhibits low toxicity and immunogenicity, has also been utilized to mask the immunogenic epitopes in lieu of directly altering viral capsid proteins. In this regard, PEGylation has been shown to decrease the immune response to AdV5 compared to native virus alone.[59,60]

An alternative approach to modifying or masking viral antigens is to indirectly prevent an immune response to them. This could be achieved by pharmacological inhibition of the immune system during transduction, prevention of transgene expression in antigen-presenting cells using tissue-specific promoters or through ex vivo infection of cells followed by their reintroduction into the patient.[46,53]

5 GENE THERAPY FOR PATHOLOGIES OF THE SKELETAL SYSTEM

While gene therapy has been used to address diseases caused by a single genetic mutation, it is more commonly studied in the skeletal field for its potential to treat complex pathologies including nonunion fractures and arthritis. Regardless of pathogenesis, most approaches used to date target anabolic, catabolic, or inflammatory pathways to promote healing and cure disease. In the past, the field focused investigation on secretable proteins, such as growth factors and cytokines; however, investigators have more recently begun to target intracellular mediators such signaling pathway components and transcription factors.

5.1 Bone

The extracellular matrix of bone consists of an organic component composed of type I collagen (COL1) as well as other noncollagenous proteins and a mineral component consisting largely of hydroxyapatite.[61] The synthesis and remodeling of bone is achieved by its three resident cell types: the bone-forming osteoblast, the bone-resorbing osteoclast, and the mechanosensing osteocyte. Under physiological conditions within the adult skeleton, the actions of the osteoblast and osteoclast are tightly coupled, and perturbations to this balance lead to bone loss or gain in diseases such as osteoporosis and osteopetrosis, respectively.[62]

5.1.1 Bone Healing and Osteogenesis

Issues surrounding bone loss and repair have immense clinical and economical importance. Bone fractures occur at an incidence of 6 million/year in the United States alone, and roughly 10% are nonunion requiring an orthopedic graft.[63] Though the gold standard for skeletal grafting therapies is the autograft, this procedure has been hindered by problems with inadequate tissue supply and donor site morbidity.[64] A solution for tissue availability has been to use allografts; however, these display reduced biological activity and mechanical properties with a potential for disease transmission.[65]

Fracture (or bone) healing is a highly complex process governed by an intricate network of mechanical and cellular factors. Primary fracture healing resembles normal bone remodeling and occurs when the fracture gap is minimized with sufficient stabilization. However, most fractures seen clinically do not stabilize completely and repair via secondary bone healing—a multistage process involving formation of a hematoma, inflammatory cell infiltration, and generation of a fibrocartilage callus that mineralizes and is subsequently remodeled until the bone is completely repaired.[66] To assess efficacy for bone healing within the preclinical milieu, investigators have utilized in vivo and ex vivo approaches to evaluate TGs for their potential to form ectopic bone and heal critical-sized defects of the calvaria and femur. More relevant to patients, models of nonunion fracture are also employed to assess the role of these factors in repair.

Of the anabolic strategies tested for bone healing, bone morphogenetic protein (BMP) family members remain at the forefront given their central role in osteoblast differentiation as well as skeletal development, remodeling, and repair.[67,68] Using nonviral and viral vectors, convincing evidence exists demonstrating that BMP-2 is capable of enhancing osteogenesis, vascularization and bone repair in calvarial, femoral, and fibular defect models. Studies utilizing nonviral vector approaches have shown that combining untransfected MSCs with BMP-2 plasmid (pBMP-2) DNA in scaffolds has positive but marginal effects on osteoblast differentiation and ectopic mineralization in vivo,[69,70] and is comparable to the effects of recombinant BMP-2 protein.[71] In addition, MSCs transfected with pBMP-2 DNA undergo osteoblast differentiation in vitro and produce ectopic bone when implanted on apatite-coated silk scaffolds in immunodeficient mice.[72]

AdV has been the most common viral vector for BMP-2-mediated gene therapy of skeletal pathologies. Direct injection of AdVs expressing BMP-2 has been shown to increase bone formation in critical-sized bone defects in rats and rabbits.[73] In another study, intramuscular injection of BMP-2-expressing AdV induced ectopic bone formation in mice, with more robust osteogenic responses observed in immunodeficient animals.[74] However, despite evidence for success of in vivo BMP-2 gene therapy, most investigations employ ex vivo approaches using MSCs and other cell types with or without scaffolds. In this regard, BMP2-expressing MSCs transduced using viral vectors significantly accelerate bone healing in various animal models.[75–78] Further, genetically modified MSCs and adipose-derived stem cells (ADSCs) expressing BMP-2 loaded onto scaffolds composed of materials, such as calcium phosphates,[79,80] devitalized bone matrix[81,82] or collagen[83,84] have been used to promote bone healing in vivo with varying degrees of success.

Stem cells are an attractive cell source for bone repair as they have the potential to directly contribute to healing. Though studies have indicated that MSCs transduced with BMP-2 transgenes may directly participate in bone repair, work done by Pensak et al. suggests that BMP-2-expressing MSCs also promote healing by recruiting resident progenitors to the defect site.[77] This finding suggests that cellular identity and differentiation potential may not be critical for ex vivo BMP-2 gene therapy. In this regard, fibroblasts transduced with BMP-2 using AdV promote healing when compared to control cells both in femoral and fibular critical-sized defect models.[85,86] Moreover, autologous muscle and adipose grafts activated to express BMP-2 by AdV transduction promote osteogenesis and bone repair in vivo.[87–89] It is important to consider that BMP-2 may drive osteogenic differentiation even in these terminal cell types and tissues as has been shown previously for fibroblasts.[90,91] On the other hand, Zwingenberger et al. demonstrated that fat tissue grafts expressing BMP-2 mediate repair through recruitment of endogenous MSC populations.[92] In either case, the use of tissues, such as muscle for delivery of TGs is advantageous given that expansion of isolated cells can be avoided and autologous tissue can be utilized.

In addition to BMP-2, a number of studies have evaluated the potency of other BMPs in mediating bone repair following injury. BMP-4-expressing MSCs healed critical-sized defects in rats,[93] with evidence suggesting that adipose-derived MSCs rather than bone marrow-derived MSCs produce more extracellular matrix in response to BMPs.[94] Muscle-derived MSCs expressing BMP-9 also promoted bone healing in a segmental bone defect model,[95] and use of scaffolds have been reported to enhance the osteogenic effect.[96] Though Betz et al. showed only a marginal effect of muscle fragments transduced with BMP-7 on femoral defects in rats,[97] an independent study using mesoporous bioglass/silk fibrin scaffolds loaded with AdV expressing BMP-7 enhanced bone formation in an ovariectomized femoral defect model.[98] In a direct comparison of BMP-2 and BMP-6, Mizrahi et al. demonstrated that both adipose- and bone marrow-derived MSCs transfected with pBMP-6 DNA generated more bone at an accelerated rate compared to BMP-2.[99] Further, plugs of protease-solubilized collagen (termed atelocollagen) loaded with pBMP-12 led to better repair of femoral bone defects than either pBMP-2 or plug alone.[100] Future comparisons of different BMPs and their effects on osteogenesis and bone healing in vivo may provide important insights for future clinical trials.

The osteogenic potential of a number of other growth factors and morphogens have also been investigated in the context of bone healing. The canonical Wnt/β-catenin pathway is essential for skeletal development and bone remodeling.[67,101] In this regard, Gao et al. found

that RV-mediated Wnt10b overexpression increased cell proliferation, BMP-2 expression, and bone mineral density leading to complete repair in a nonunion atrophic fracture model.[102] Fibroblast growth factor 2 (FGF-2) has also demonstrated efficacy in calvarial defect models, where pFGF-2 transfected bone marrow-derived MSCs embedded on a hydroxyapatite scaffold accelerated healing compared to controls.[103] In an independent study, genetically modified MSCs overexpressing FGF-2 by AAV transduction enhanced blood vessel and bone formation in calvarial defects.[104]

Vascularization of the fibrocartilage callus is critical for bone formation and fracture healing. As such, a number of studies have used the potent angio- and vasculogenic inducer vascular endothelial growth factor (VEGF) to enhance bone repair; however, use of VEGF alone as a gene therapy has been met with mixed results. For instance, genetically modified BMSCs expressing VEGF loaded onto collagen-modified PLGA/TCP scaffold promoted vascularization and new bone formation in a critical-size defect model of the distal radius.[105] On the other hand, though RV delivery of VEGF cDNA to human MSCs on silicate-substituted apatite granules increased vascularization of grafts, they showed less bone compared to untransduced controls.[106] Despite the conflicting evidence for VEGF monotherapy, combined expression of VEGF with BMP-6 enhances osteogenesis compared to BMP-6 alone, and treatment with VEGF and BMP-2 promotes vascularization without negatively affecting BMP-2-induced bone formation in vivo.[76,107]

Cyclooxygenase-2 (COX-2) regulates synthesis of prostaglandins and is required for chondrogenesis and cartilage formation during fracture healing.[108,109] As a therapeutic target for gene therapy, in vivo delivery of COX-2 via RV to periosteal cells following fracture led to increased MSC recruitment to the fracture callus, decreased cartilage formation, and enhanced angiogenesis and remodeling of the cartilage fracture callus.[110] This study was followed by a second that used microarray to reveal that direct delivery of RV expressing COX-2 to the fracture site results in downregulation of inflammatory genes and upregulation of transcriptional regulators of hematopoiesis and erythropoiesis.[111] A previous study from the same group had demonstrated that COX-2 gene transfer suppressed repair of calvarial defects;[112] however, calvarial bones heal without the cartilaginous callus seen in the long bones. Therefore, given that COX-2 regulates fracture healing by modulating chondrogenesis and vascular remodeling, its use as a gene therapy may only be suitable for repair of long bone defects.

In addition to targeting extracellular mediators, recent studies have focused on directly modulating intracellular signaling pathways. For instance, overexpression of the osteoblast master regulator runt-related transcription factor 2 (Runx2) in ADSCs delivered to radial bone defects or sites of mandibular distraction promotes osteogenesis and bone healing in vivo.[113,114] Furthermore, combinatorial expression of Runx2 with BMP-2 in ADSCs using a bicistronic vector enhanced in vitro osteoblast differentiation and ectopic bone formation in athymic rats compared to cells transduced with BMP-2 alone.[115] In addition to transcription factors, miRNAs have been adopted for use in gene therapy for bone repair. For instance, delivery of miR-26a mimics using transfected BMSCs led to complete repair in a calvarial defect model.[116] Additionally, a separate study found that RV-mediated delivery of miR-148b together with BMP-2 to ADSCs promotes repair of calvarial defects in mice.[117] In other studies, knockdown of miR-214 promotes repair of femoral defects in ovariectomized (OVX)-rats where BMP-2 alone failed.[118] Further, use of antisense sequences to knockdown miR-31 in BMSCs loaded onto bioglass scaffolds promotes osteoblast differentiation and healing of calvarial critical-sized defects.[119]

5.1.2 *Implant Stability and Aseptic Loosening*

Successful hip arthroplasty or dental implant placement requires that the implant surface integrates with the bone (referred to as osteointegration). Aseptic loosening typically precedes failure of the implant, which is characterized by the generation of tiny particles at the interface between bone and the implant surface. This debris may provoke granuloma formation, bone resorption, or inflammatory cell infiltration leading ultimately to osteolysis and loss of the prosthesis.[120]

The molecular mechanisms that contribute to failed osteointegration and aseptic loosening remain unclear. As aseptic loosening progresses, the appearance of the implant/bone interface shares some histological characteristics with rheumatoid arthritis (RA) and a foreign body reaction.[121] In particular, IL-1 and TNFα have been implicated as potent mediators to implant failure as both these chemokines can trigger downstream immune responses and osteoclastogenesis. Specifically, osteoclastic modulatory factors osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL) are upregulated around failing implants and the increased RANKL:OPG ratio favors bone resorption.[120]

Retroviral vectors encoding human IL-1 receptor antagonist (IL-1Ra) and soluble human tumor necrosis factor receptor (sTNF-R) have been evaluated using an air pouch model, in which articulate wear debris was coimplanted with bone substrate into the subcutaneous space.[122] In this study, IL-1Ra gene transfer decreased inflammation and pouch fluid accumulation while simultaneously lowering macrophage infiltration. In vivo gene transfer of OPG using either AdV or AAV has also been employed to target osteoclasts directly. In this regard, AAV expressing OPG exhibited protective effects against orthopedic wear in two different murine models of osteolysis,[123] and the expression of

OPG reduced bone calcium release by approximately 40% compared to placebo-injected groups. In addition, overexpression of OPG by AdV at the bone defect site prior to placement of titanium implant was found to reduce osteoclast number, increase osteointegration and improve implant stability.[124] A combinatorial approach in which OPG and IL-1Ra were delivered to the peri-implant site by AAV and RV, respectively also decreased wear-induced osteoclastogenesis and increased the force required for implant extraction.[125]

5.1.3 Osteoporosis

Osteoporosis remains the most prevalent low bone mass disease both in the United States and worldwide.[126] In osteoporotic patients, bone mass is decreased leading to bone fragility and increased fracture risk. At the cellular level, this disease is caused by an imbalance in bone remodeling resulting from decreased bone formation and excessive bone resorption. The most prevalent therapeutic strategies utilize pharmacological agents that inhibit osteoclastogenesis and bone resorption. Teriparatide is a recombinant form of parathyroid hormone (PTH) 1–34 and remains the only FDA-approved anabolic agent for treatment of osteoporosis, though other biologics targeting Wnt signaling are in various stages of clinical trials.[127]

In the past 5 years, investigators have begun to use gene therapy to increase bone mass either in wild-type mice or preclinical models of osteoporosis. Using an in vivo nonviral approach, one study demonstrated that systemic hydrodynamic injection of insulin-like growth factor 1 (IGF-1) cDNA into the tail vein of OVX-mice increased serum and histomorphometric markers of bone formation and bone mineral density.[128] An alternative approach has been to use stem cell antigen-1-positive (Sca-1+) hematopoietic progenitors to deliver TGs to the bone microenvironment. These cells are particularly useful for systemic as well as local injection given their ability to migrate and engraft to the bone marrow niche. In this regard, Hall et al. in 2007 demonstrated that retro-orbital delivery of genetically modified Sca-1(+) cells expressing a modified FGF-2 transgene into sublethally irradiated mice lead to increased serum levels of FGF-2 and bone formation markers associated with extensive endosteal bone formation.[129] However, a number of adverse effects were also observed, including partial or complete loss of the bone marrow space, osteomalacia, and hyperparathyroidism. A follow-up study by the same group utilized the erythroid-specific promoter β-globin in place of a ubiquitous viral promoter to express the modified FGF-2 transgene in Sca-1(+) cells. In this context, the authors saw extensive bone formation in the endosteum of injected mice with no evidence of osteomalacia and less pronounced hyperparathyroidism.[130] Similar results have also been observed with platelet-derived growth factor subunit B (PDGFB).[131] In a separate approach, intramedullary injection of a RV-siRNA targeting the adipocyte critical transcription factor peroxisome proliferator-activated receptor γ (PPARγ) increased bone mass, osteoblast number while decreasing the number of osteoclasts.[132] Taken together, these recent advances suggest that gene therapies may one day exist for the treatment of systemic bone diseases, such as osteoporosis.

5.1.4 Osteogenesis Imperfecta

Osteogenesis imperfecta (OI) is a genetic connective tissue disease that occurs with an incidence of one in every 10,000–20,000 live births.[133] While poor bone quality is the predominant feature of OI, other tissues rich in COL1 including tendons, ligaments, and teeth can also be affected. The disease shows significant clinical variability with dominant and recessive inheritance being linked to mutations in *COL1* or its posttranslational modification machinery, respectively. Strategies to limit fracture and increase bone mass in patients typically employ pharmacological agents, such as bisphosphonates to inhibit osteoclastic activity. At the same time, emerging evidence suggests that increasing Wnt signaling or inhibiting transforming growth factor β (TGF-β) may improve bone mass and strength in mouse models of dominant and recessive OI.[134,135]

It has been suggested that OI patients with gain-of-function mutations in *COL1* could benefit from ex vivo gene therapy designed to reduce COL1 levels. Certainly, in an in vitro context, Millington-Ward et al. have shown that using the hammerhead ribozyme Rzpo1a1, which targets a common *COL1A1* polymorphism, they could downregulate *COL1A1* transcripts at a ribozyme to transcript ratio of 1:1 in human MSCs;[136] however, it is unclear whether this strategy would work in vivo. A second approach might be to transplant MSCs transduced with wild-type *COL1* into OI patients with the hopes of replacing aberrant COL1 with the wild-type protein. In this regard, it has been shown that *Col1a1* from wild-type MSCs infused into transgenic OI mice expressing a human mini-COL1A1 could be detected in various tissues including marrow, cartilage, and bone.[137] Further, at 1-month postinfusion, humeri from OI-transgenic mice that received wild-type MSCs displayed modest increases in collagen and mineral content.[137] That being said, gene therapies targeting dysregulated cell signaling caused by extracellular matrix disruptions (as opposed to the extracellular matrix itself) may be more advantageous and widely applicable given recent successes using anti-TGF-β antibody therapy.[135]

In recent years, mutations in genes other than *COL1A1* or *COL1A2* have been identified as causing recessive forms of OI. For instance, mutations in *SERPINF1*, a gene that encodes pigment epithelium-derived factor (PEDF), have been identified in patients with OI type VI.[138,139] Given that PEDF is a secreted glycoprotein

protein, Rajagopal et al. investigated whether restoration of circulating PEDF in the blood could rescue the low bone mass phenotype seen in *Serpinf1*$^{-/-}$ mice.[140] Surprisingly, though HDV-mediated overexpression of PEDF within the liver restored serum levels of the protein, it failed to correct the bone phenotype in this mouse model. At the same time, overexpression of PEDF in vitro did increase osteoblast differentiation and matrix mineralization of an LV-transduced osteoblast-like cell line,[140] suggesting that PEDF may need to be produced locally within bone itself.

5.2 Cartilage

Most gene therapies for cartilage have focused on treatment of either osteoarthritis (OA) or RA—the two major diseases of articular joints. Articular joints consist of a diverse array of tissues including cartilage, synovium and synovial fluid, subchondral bone, menisci, ligaments, muscles, and nerves. The extracellular matrix of articular cartilage, which is both avascular and aneural, is composed primarily of type II collagen (COL2) and proteoglycans, and is produced and maintained by resident chondrocytes. During movement, articular cartilage functions to reduce friction between articulating skeletal elements and protect the subchondral bone from mechanical stress.[141] Though changes to articular cartilage play a significant role in pathogenesis of joint degeneration, the synovium, subchondral bone, and menisci must also be considered when developing therapies for these multifactorial diseases.

In contrast to conventional recombinant protein therapies for joint disease, gene therapy has the potential to deliver appropriate concentrations of a therapeutic agent in a localized and sustained manner. A number of vectors and transgenes have been evaluated and shown to have varying degrees of efficacy in different animal models of OA and RA. Although the first clinical trials were performed with RVs, researchers have shifted preference more recently to the safer and easier-to-produce AdVs and AAVs. In this regard, HDV transduces chondrocytes at a higher efficiency compared to AAVs, and expression of genes delivered using HDV can be sustained for over a year following intraarticular delivery.[142]

Of the cell types present in synovial joints, synoviocytes are preferentially targeted by most viral vectors including HDVs. When dealing with secreted factors that act extracellularly on joint tissues, transduction of synoviocytes may be sufficient; however, their high rate of turnover in OA and RA can lead to reduced TG expression overtime. Given that chondrocytes exhibit slower turnover during OA pathogenesis (particularly in early stages of disease), they may be a more relevant target for gene therapy when using nonintegrating viral vectors. However, chondrocytes do not express CAR, the major AdV receptor, and while transduction using these vectors can be achieved, it requires much higher doses than other tissues. To overcome this obstacle, Ruan and colleagues conjugated a monoclonal antibody against α-10 integrin (a10mab) to a HDV with biotin acceptor peptide-modified fibers. α-10 integrin is the surface receptor forCOL2 and is expressed by chondrocytes of the joint. In this study, the authors demonstrated that these fiber-modified vectors could successfully retarget the TG of interest to chondrocytes leading to 10-fold reduction in effective dosage[143] (further details later).

5.2.1 Osteoarthritis

OA is a major cause of disability and one of the most common musculoskeletal disorders, with its economic burden on the US health care system alone estimated to exceed $100 billion annually.[144] This degenerative disease of the joint is characterized by loss of articular cartilage, periarticular and subchondral bone remodeling, intraarticular inflammation with synovitis, and pain.[145] To date, no medical therapies are available that markedly alter OA pathogenesis. Instead, current interventions are limited to symptomatic relief of pain, physical therapy and, in end-stage disease, joint replacement.

Articular cartilage displays a limited potential for self-regeneration. As such, anabolic agents such as TGF-β,[146–148] IGF-1,[149–151] FGFs,[151–153] and BMPs (most recently BMP-2 and BMP-4)[147,154–156] have been attractive therapeutic targets. For instance, Mi et al. injected an AdV encoding human IGF-I into arthritic rabbit knee joints and found it was able to maintain a healthy hyaline cartilage phenotype by promoting matrix synthesis without evidence of chondrocyte hypertrophy.[157] However, the joint is composed of other tissues apart from articular cartilage that may be adversely affected by growth factor gene therapy. In this regard, though TGF-β1 acts on chondrocytes to drive cartilage matrix synthesis, its overexpression within the joint causes fibrosis and formation of cartilaginous nodules within the synovial membrane.[158] Taken together, careful dosage tests will be necessary for any anabolic growth factor before clinical trials can proceed, and the effects of various therapeutic interventions on all tissues in the joint must be considered.

Recent work in this field has also focused on using transcription factors, such as the RUNX2,[44] and the cartilage master transcription factors sex-determining region Y (SRY)-box 5 (SOX-5), SOX-6, and SOX-9 (SOX trio)[159,160] as anabolic agents for gene therapy. In this regard, Needham et al. generated gene-activated bilayered scaffolds with an upper layer impregnated with vectors carrying the SOX trio to activate chondrogenesis and a lower layer carrying vectors of RUNX2 to stimulate osteogenesis. The study showed that, contrary to previous studies in which cells were simply cotransfected with the transcription factors, the spatially-distributed combination led to

greater tissue ingrowth and implant degradation.[44] Sieker et al. also utilized implants of autologous bone marrow coagulates transduced with indian hedgehog (IHH), a transcription factor critical for chondrocyte proliferation and maturation, in an osteochondral defect model in rabbit knees. Interestingly, this study showed superior histological repair compared to BMP-2- and green fluorescent protein (GFP)-transduced groups, including improvements in COL2 staining, tissue morphology, and surface integrity.[155]

In addition to deficits in anabolism and tissue regeneration, OA is marked by progressive inflammation and tissue degradation. To address this issue, a plethora of anti-inflammatory and anticatabolic targets have been tested in recent years. While previous work focused primarily on blockade of the IL-1 pathway through use of IL-1Ra,[152] alternative targets and approaches have become more widely investigated. One exciting new trend is the use of siRNA to silence catabolic and/or inflammatory genes.[161,162] Hypoxia-inducible factor-2α (Hif-2α) controls the hypoxic response and has been shown to directly induce the chondrocytes to express a wide variety of catabolic factors. It is also upregulated in OA cartilage where it is thought to play a central role in articular cartilage destruction. Using a chondrocyte-targeting peptide carrying anti-Hif-2α siRNA to knockdown Hif-2α, Pi et al. observed that a multitude of catabolic genes were downregulated, including matrix metalloproteinases (MMPs-13 and -9), aggrecanse-1, as well as other markers of cartilage degradation, such as type X collagen (COL10) and VEGF. When injected into knee joints of mice with surgically induced OA, anti-Hif-2α siRNA improved cartilage protection as demonstrated by decreased Mankin scores, reduced synovitis, and reduced IL-1β.[163]

Components of the extracellular matrix and synovial fluid have also received attention in OA-related gene therapy in recent years. For instance, Ruan et al. showed that both genetic and HDV-mediated overexpression of lubricin (PRG4) in the knee joint significantly delayed disease progression in posttraumatic and age-related models of OA.[142] PRG4, a protein secreted by superficial zone chondrocytes and synovial lining cells, was first described as a chondroprotective factor that provided synovial fluid with the ability to dissipate strain energy produced by loading.[164] In addition to its reported role in joint lubrication, Ruan and coworkers found that PRG4 overexpression could inhibit expression of cartilage catabolic and hypertrophic genes through upregulation of Hif-3α.[142] Moreover, the therapeutic efficacy of PRG4 gene therapy could be enhanced using a fiber-modified HDV that retargeted viral vectors to chondrocytes.[143]

Though many of the approaches described earlier have led to promising therapeutic outcomes in animal models of OA, it is unlikely that this complex disease will respond long term to a single TG; as such, combinatorial therapies have become a focus in this field. While some investigators have focused efforts on testing different growth factor pairs,[153] others have explored combinations of anabolic, anticatabolic, and/or antiinflammatory targets[149,152] with varying degrees of success. For instance, Madry et al. used alginate to deliver NIH-3T3 cells transfected with expression plasmids for either IGF-1 alone or IGF-1 and FGF-2 into osteochondral defects in rabbits, and saw improved articular cartilage repair and enhanced subchondral osteogenesis at the defect site when IGF-1 and FGF-2 were used in combination.[153] Taken together, a combinatorial approach (together with advances in tissue engineering) is likely to have the best chance at effectively treating disease.

5.2.2 Rheumatoid Arthritis

RA affects approximately 1.3 million people in the United States alone; however, despite its economic burden, effective treatment options remain limited and no cure is available. Unlike OA, RA is an autoimmune disorder characterized by a chronic inflammatory state within the joint that leads to cartilage degradation, bone damage, pain, and disability that often present with or are followed by other chronic systemic health conditions, such as cardiopulmonary disorders.[165] Though a complete understanding of the pathogenicity of RA is yet to be elucidated, it is known that there is an interplay of genetic susceptibility and environmental factors leading to a loss of self-tolerance, the development of autoantibodies, and ultimately joint destruction.[166]

There is a greater diversity of targets being investigated clinically for RA gene therapy compared to OA. The first clinical trials for RA gene therapy began in 1996, and others have followed.[167] Still, despite the variability in therapeutic targets, a large percentage of the focus continues to be on antiinflammatory factors, such as IL-10,[168–171] binding immunoglobulin protein,[172] and tumor necrosis factor receptor type II (TNFR-II). Inhibition of TNF-α has shown promising results, which led to FDA approval of TNF-α monoclonal antibodies (infliximab and adalimumab) and a TNF-α soluble receptor antagonist (etanercept). Unfortunately, these TNF-α inhibitors must be administered frequently to maintain therapeutic concentrations within the joint—a need that could be easily met using a gene therapy approach. In this regard, a number of studies have demonstrated the feasibility of anti-TNF-α gene therapy strategy using AdV, RV, and AAV.[173,174] For instance, Chan et al. demonstrated in a streptococcal cell wall rat arthritis model that local (intraarticular) as well as systemic (intramuscular) AAV-mediated expression of TNFR-II fused to the Fc domain of immunoglobulin IgG_1 (TNFR:Fc) decreased inflammatory cell infiltration, pannus formation, cartilage and bone destruction. Furthermore, studies with an AAV expressing TNFR:Fc under the control of the

inflammation-inducible NF-κB promoter demonstrated the possibility of having TG expression specifically during disease flare-ups. This approach is particularly advantageous as it would avoid unwanted expression of immune suppressive agents during disease regression in RA patients. Unfortunately, a 2008 clinical trial of AAV2-TNFR:FC (termed tgAAC94) was halted early when a 36-year-old female trial enrollee succumbed to an infectious disease shortly after receiving her second dose. Postmortem analysis determined that the patient had died from a *Histoplasma capsulatum* fungal infection, a known complication for patients taking TNF antagonists. Since the patient was also taking adalimumab concurrently with the study, it is difficult to determine the specific role, if any, tgAAC94 played in her death. Patient-reported outcome measures showed improvement in 42% of AAV2-TNFR:Fc cases versus 19% in the placebo group. Though the FDA has since cleared the study organizers of wrong doing and allowed the trial to continue, no phase III trial has been scheduled as of yet; in addition, the developing company has changed its name and removed tgAAC94 from the list of pipeline candidates.[175] Another key proinflammatory cytokine induced during RA pathogenesis is Il-1β. The endogenous Il-1Ra can inhibit the effects of Il-1β in inflammatory conditions and has displayed efficacy in various RA models. Consequently, recombinant Il-1Ra has been developed and was approved by the FDA in 2001 for treatment of RA. Given the short in vivo half-life of recombinant IL-1Ra (~4 h), several investigators have begun to test the efficacy of various viral vectors for sustained delivery of IL-1Ra to the joint. For instance, synoviocytes transduced ex vivo with RV-expressing Il-1Ra prevented disease progression in mouse and rabbit RA models.[176,177] Also, direct intraarticular injection of an Il-1Ra-expressing AdV abrogated disease activity in an autoimmune arthritis mouse model. Finally, other groups have attempted to directly modulate the immune response by targeting Th-17 cells[178–180]

While inflammation is a logical target for treatment of RA, some groups are attempting to address other aspects of disease pathogenesis. The aggressive, tumor-like proliferation of fibroblast-like synoviocytes during RA progression has led to testing of tumor-suppressive agents. Specifically, the rapid expansion of fibroblast-like synoviocytes is known to be triggered by activation of phosphatidylinositol 3-kinase (PI3K)/AKT and Ras/extracellular signal-regulated kinase (ERK) signaling pathways, which results in massive upregulation of inflammatory cytokines and MMPs. Zhang et al. attempted to alter this proliferative and catabolic phenotype by overexpressing Sprouty2 (SPRY2), a known inhibitor of the PI3K/AKT and Ras/ERK cascades.[181] In these experiments, rats were treated with AdV-SPRY2 (or AdV-GFP) 7 days following induction of adjuvant-induced arthritis. The authors observed that joint swelling and rigidity were reduced in mice treated with AdV-SPRY2 compared to the controls; further, proinflammatory cytokine production, synovial hyperplasia, and cartilage degradation were diminished.[181]

In addition to synovitis, RA is marked by extensive bone loss owing to overactivation of osteoclasts. As such, a number of recent investigations have looked at modifying disease progression by suppressing osteoclast activity through overexpression of OPG.[182,183] In this regard, Zhang et al. injected collagen-induced arthritis rats with an AdV-OPG at both early and established arthritis time points. The authors determined that cartilage degradation enzymes, proteoglycan loss, and chondrocyte apoptosis were inhibited by the treatment; however, overall cartilage destruction was only improved in the early treatment group. Additionally they found, in alignment with previous studies, that the treatment had no impact on swelling or inflammation.[182] Thus, similar to findings for OA, this study further emphasizes the importance of combinatorial treatments for modification of joint disease pathogenesis.

5.3 Tendon

Tendons are viscoelastic tissues that function to transmit force from muscular contractions to the skeleton.[184,185] The extracellular matrix of tendon is composed primarily of COL1 as well as smaller quantities of other collagens, elastin, and proteoglycans.[186] Following injury, tendons heal incompletely leading to formation of scar tissue and inferior biomechanical properties compared to uninjured tissue. In addition to partial and complete tears, tendons often rupture at their insertion into bone (termed the enthesis). Further, tendons can develop adhesions, which interferes with gliding leading to compromised function.

Similar to strategies for bone healing, the gene therapy field has targeted a variety of growth factors and intracellular signaling mediators to improve tendon repair. BMP-12 has been found to induce transformation of soft tissue into ligament- or tendon-like tissue,[187] and transduction of primary chicken tendon in vitro with AdV expressing BMP-12 induced Col1 synthesis.[188] Moreover, in an in vivo model of complete flexor digitorum profundus laceration, injection of an AdV expressing BMP-12 led to significantly greater ultimate failure force and stiffness compared to controls. Similar to BMP-12, BMP-13 overexpression has been shown to drive ligamentous differentiation in MSC cultures.[189] Moreover, in vivo BMP-13 gene therapy promoted tendon healing more effectively compared to platelet-rich plasma (contains growth factors, such as TGF-β) and control AdV in an injured rat supraspinatus model.[190] In addition to BMPs, delivery of pIGF-1 DNA to wounded rat Achilles

tendon improved histological and biomechanical properties during healing compared to controls, suggesting that IGF-1 may be an interesting candidate for healing of midtendon injury.[191]

Apart from midtendon damage, failure following excessive or repetitive loading often occurs at the enthesis. Interestingly, osteogenic factors with proven efficacy in bone healing following fracture also enhance repair at this site. For instance, a rat cell line transfected with pBMP-2 mixed with calcium alginate gel and implanted within the tunnel formed during tendon grafting improved osteointegration and vascularization compared to control.[192] With respect to intracellular targets, application of RV-expressing COX-2 during surgical repair of the tendon enthesis enhanced angiogenesis, osteointegration, and pull-out strength of the tendon graft.[193] Further, two separate studies examining in vivo Sox9 and ex vivo Scx (tendon master transcription factor) gene therapies demonstrated improved biomechanical strength at the enthesis with minimal alterations to histological appearance compared to controls.[194,195]

While gene therapy approaches to improve healing of tendon injuries have shown promising results, only very few TGs in a limited number of studies have been evaluated. With increased understanding of the underlying molecular biology of tendon development and disease, it is the hope that more gene therapy targets for ligament and tendon healing might be identified in the future.

5.4 Intervertebral Disc

Intervertebral discs (IVD) are elastic structures found between vertebrae composed of the annulus fibrosis on the exterior and the nucleus pulposus (NP), its inner gel-like center. The NP is avascular with its extracellular matrix composed primarily of proteoglycans and COL2, and serves as a pressure distribution pad for the spinal column. During IVD degeneration, the size of the NP decreases leading to increased strain on the annulus fibrosis, which increases in size to compensate. The extracellular matrix of the NP condenses and is slowly replaced with a more fibrous tissue. All of these changes serve to alter the biomechanical properties of the disc and is one of the leading causes of lower back pain.[196]

As in OA, much attention has been paid to targeting growth factors to improve regeneration, including the TGF-β superfamily,[197] growth and differentiation factor 5 (GDF-5),[198] and IGF-1.[199] The goal of treatment with growth factors is to induce proliferation of NP resident cells, reduce apoptosis, and induce extracellular matrix production (proteoglycans, COL2, and aggrecan).[200] The BMP family is also a common target, with labs investigating BMP-2[201] and BMP-7.[202]

Similar to gene therapy approaches for OA and RA, combinatorial trials using anabolic and anticatabolic targets are becoming more common in this field. Liu et al. tested the in vitro combination of connective tissue growth factor (CTGF) as an anabolic agent and tissue inhibitor of metalloprotease-1 (TIMP-1) as an anticatabolic. CTGF promotes the expression of matrix components and inhibits apoptosis, while TIMP-1 inhibits MMP production. They found that the combination of the two factors increased production of proteoglycans and COL2 over either monotherapy.[203] Yue et al. tested in vivo the combination of survivin, TGF-β3, and TIMP-1 in a rabbit model of IVD disease and found that the combinatorial injection slowed progression of degeneration compared to their empty vector treatment groups. It should be noted though that they did not include any single therapy controls to determine if the combination is empirically better than any single target alone.[204] It is likely that survivin was included in the combination as it is upregulated in cells of patients with degenerative disc disease and knockdown via siRNA in vitro reduced NP cell proliferation and sensitized them to apoptotic stimuli in vitro;[205] however, other groups have shown that overexpression of survivin had no impact in vitro on cell survival.[206]

One target under investigation in this field that is unique from targets being actively investigated for arthritis is the telomerase reverse transcriptase (TERT). TERT is found in stem cells, and was initially used to prolong the activity and expansion of NP cells grown in culture to permit greater production of NP cells for transplantation.[207] Shi et al. have shown that these transduced hTERT-NP cells are also superior to nontransduced NP cells in a dog model as they delay degeneration and preserve structural integrity, matrix composition, and mechanical stability.[208] It will be interesting to see if combinatorial therapy using hTERT-NP cells together with other targets, such as GDF-5 or TIMP-1 can improve upon these results.

6 CONCLUSIONS

Gene therapy has many characteristics that could contribute to improvements in current treatments of skeletal diseases. Importantly, gene therapy confers stable, long-term expression of a therapeutic protein, which otherwise would have to be frequently administered. Many gene therapy approaches have been evaluated in vitro and in vivo in small and large animal models, and a few have been tested in human clinical trials. Due to the wealth of preclinical data, researchers have insight about which TGs might be most useful for certain conditions. Moreover, given the immense evaluation and optimization of various vectors in both in vivo and ex vivo contexts, researchers can now choose the most suitable gene transfer approach for their therapeutic needs. Although the number of clinical trials is still limited, data show that gene therapy for skeletal diseases is safe especially when

ex vivo or local in vivo gene therapies are used. No gene therapy agents have so far been approved by regulatory agencies in the United States; however, Glybera (a treatment for lipoprotein lipase deficiency) was approved in Europe in 2012 and two gene therapy drugs for cancer are on the market in China. More recently, Strimvelis, the first ex vivo stem cell gene therapy for treatment of adenosine deaminase severe combined immunodeficiency, was approved by the European Commission in May 2016. At present, the gene therapy treatments that are the most likely to receive approval treat rare life-threatening diseases, whereas treatments for complex, less life-threatening diseases, such as those discussed here may take more time to gain approval as the regulatory agencies become more comfortable with the concept. The development of safer vectors with long-term expression, such as HDVs and AAVs, and improvements in technologies, such as gene-activated scaffolds, should facilitate the next wave of clinical trials in both ex vivo and in vivo contexts.

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CHAPTER

9

Pharmacogenetics and Pharmacogenomics of Osteoporosis: Personalized Medicine Outlook

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Osteoporosis and its consequence of fragility are recognized as a major skeletal disorder, because they are associated with increased risk of mortality and major healthcare costs. The disease affects up to 40% postmenopausal women and 15% elderly men of Caucasian background.[1] From the age of 50, approximately 44% of women and 25% of men will sustain a fracture during their remaining lifetime.[2] Although any bone can sustain a fracture, the residual lifetime risk of hip fracture, often considered the most serious fracture is 8.5% for women and 4% for men.[2] Hip fracture, along with most osteoporotic fractures, is associated with increased risks of subsequent fractures,[3] premature mortality,[4,5] and incurs significant health care cost.[6,7] Moreover, up to 24% women and 38% men will die within the first 3 months after experiencing a hip fracture.[8,9] All types of fragility fracture are associated with loss of independence, loss of quality of life, and premature mortality.[10–12] Survivors after fragility fractures often develop chronic pain, decreased independence, and/or reduced quality of life. Taken together, fragility fracture is a common and serious bone disorder that is expected to increase in magnitude over the next few decades as populations are rapidly aging.

In osteoporosis care, a much anticipated application of the Human Genome Project (HGP) is the translation of genetic discoveries for early identification of high-risk individuals and selection of appropriate therapies for an individual. Osteoporosis offers an interesting case for the application of genetics in risk prediction, because the susceptibility to the disease is determined by genetic and environmental factors. Moreover, osteoporosis is a condition characterized by multiple phenotypes, including worsened bone strength and architecture, and ultimately fragility fracture. Currently, there are multiple treatments and combination of treatments available for reducing bone loss and reducing fracture risk. These are effective in some, but not all individuals. The efficacy and clinical outcome of current therapies are highly variable among patients. How to assess the risk of fracture for an individual (i.e., individualized prognosis), and which treatment is appropriate for an individual (e.g., individualized treatment decision) are among the most pressing questions in osteoporosis research. Pharmacogenetics and pharmacogenomics offer the possibility to individualize fracture prognosis and therapy.

Pharmacogenetics refers to the science of how genetic factors affect the interindividual variation in drug efficacy and safety.[13] A goal of pharmacogenomics is to identify for all human genes, their products, interindividual variation, intraindividual expression, and function over time.[14] The ultimate aim of pharmacogenomics is to select the right treatment for an individual patient and to design new drugs. In reality, most pharmacogenetic studies focus on single genes and their associations with interindividual differences in drug behaviors, while pharmacogenomics is concerned with the genomic interactions among genes in the overall variation in drug metabolism and response. Both pharmacogenetics and pharmacogenomics have important applications in studies of the pathogenesis and treatment of osteoporosis.[15–17]

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1 COMPLEXITY OF PHENOTYPES

Osteoporosis has multiple phenotypes, and this presents a challenge for the genetic dissection of the disease. Fracture is a direct consequence of bone fragility and is therefore a key component of an osteoporosis phenotype. However, fracture is a dichotomous event, resulted from cumulative deterioration in multiple tissues, which are in turn linked to genetic and nongenetic factors. Moreover, fracture is age-dependent such that the risk of fracture increases exponentially with advancing age. Theoretically, if the life expectancy of a population were infinite, then the lifetime risk of fracture would be 100%. Therefore, a genetic analysis of fracture as a single phenotype will not adequately capture the dynamics of osteoporosis.

Fracture itself is associated with multiple risk factors, some of which may be causal factors. These factors include advancing age, low bone mineral density (BMD), a personal history of fracture, fall, low body mass index, and interestingly genetic factors.[18] Recent data have shown that the 10-year probability of fracture at the forearm, humerus, spine, or hip increases between ages 45 and 85 as much as eightfold for women and fivefold for men.[19] A prior fragility fracture signals substantially elevated risk of future fracture.[4,20–22] The elevated risk is 1.5- to 9.5-fold depending on age at assessment, number of prior fractures and the site of the incident fracture. Pooling the results from all studies (women and men) and for all fracture sites, the risk of subsequent fracture among those with a prior fracture at any site is 2.2 times that of those without a prior fragility fracture.[22]

BMD, as a phenotype of osteoporosis, is causally related to fracture risk, such that there is a strong, continuous, and consistent relationship between BMD and fracture risk. Each standard deviation (SD) lower BMD is associated with a 1.6-fold increase in fracture risk in both men and women.[23] The magnitude of association between BMD and hip fracture risk (with relative risk being 2.2[24]–3.6[25]) is equivalent to or even stronger than the association between serum cholesterol and cardiovascular disease. Moreover, therapies that increase BMD reduce fracture risk.[26] Given the strong association between BMD and fracture risk, in 1994 the World Health Organization (WHO) expert panel proposed an operational definition of osteoporosis, by which a postmenopausal woman is considered to have osteoporosis if her femoral neck BMD is at least 2.5 SD lower than the mean value in young adults.[27] Nevertheless, in the same way that "hypertension" relates to cut-off value for blood pressure measurements, there is no threshold of BMD that discriminates absolutely between those who will or will not have a clinical event. Hence, a nonosteoporotic BMD does not guarantee that fracture will not occur; only that the risk is relatively lower. Conversely, if BMD is in the osteoporotic range, then fractures are more likely, but still may not occur. Low BMD alone does not completely account for the incidence of fragility fracture. More than 50% of women and 70% of men with a fragility fracture do not have "osteoporotic" BMD.[28] This suggests that factors other than BMD play important roles in the determination of fracture liability.

Biochemical markers of bone remodeling can also be considered phenotypes of osteoporosis. Bone mass is the net result of two counteracting processes of bone resorption and bone formation, often referred to as bone remodeling. Bone remodeling is a normal, natural process that maintains skeletal strength, enables repair of bone damage, and is essential for calcium homeostasis. During the remodeling process, osteoblasts produce a number of cytokines, peptides, and growth factors that are released locally and into the circulation. Their concentration thus reflects the rate of bone formation. Osteoclasts produce bone degradation products that are also released into the circulation; most of these are cleared via the kidney. These include both enzymes and nonenzymatic peptides derived from cellular and noncellular compartments of bone. It has been proposed to estimate bone formation and bone resorption by serum or urinary biochemical markers.[29] These markers include osteocalcin, bone-specific alkaline phosphatase and procollagen I N-terminal telopeptide (PINP) and collagen C-terminal propeptide of type 1 collagen (PICP) for formation and for resorption, urinary excretion of hydroxyproline, pyridinoline (PYR), deoxypyridinoline (D-PYR), collagen type I cross-linked N telopeptide (NTX) and collagen type I cross-linked C telopeptide (CTX). Markers of bone formation and resorption have been shown to be related to bone loss, with higher rates of bone resorption being associated with more rapid bone loss and importantly, with fracture risk.[30–32] However, few genetic studies of osteoporosis have analyzed bone turnover markers as phenotypes.[33,34]

Fragility fracture is also a function of nonskeletal factors, such as fall propensity that is affected by neuromuscular function, muscle strength, and postural sway.[35] Many of these nonskeletal traits are also determined by genetic factors. The liability to fracture is therefore a complex phenotype, in the sense that it is a constellation of bone strength and nonskeletal factors, and each of these factors may be determined by specific genes or sets of genes.[36]

2 GENETICS OF OSTEOPOROSIS

Through several twin and family studies, it is now clear that all osteoporosis phenotypes aggregate in families. A key measure of genetic influence on a trait is the index of heritability, which is defined as the extent to

which genetic differences contribute to individual differences in an observed trait. In a Finnish twin study, approximately 35% of the variance in the liability to fracture (in both males and females) was attributable to genetic factors.[37] In a recent family study, approximately 25% of the liability to one fracture type, that is, Colles' fracture of the wrist, was attributable to genetic factors.[38] Familial analysis within the Study of Osteoporotic Fracture[39] suggests that women, whose mother had had a hip fracture, had a twofold increase in risk of hip fracture compared with controls. The risk of hip or other fractures was threefold higher with a paternal history of wrist fracture. In two small studies of osteoporotic women with vertebral or hip fractures, their daughters had bone density deficits intermediate between their mothers and "expected" at the site of their mothers' fracture, that is, lumbar spine or proximal femur.[40,41] Similar observations have been made in both elderly men and women.[42] In summary, these biometric studies have consistently shown that the liability to fracture is partly determined by genetic factors.

BMD is one of the more heritable traits in human. Indeed, variation in BMD among individuals is largely determined by genetic factors. It has been estimated from twin studies that 70%–80% of variance of BMD measured at the lumbar spine and femoral neck is attributable to genetic factors.[43–45] The heritability of forearm BMD appears to be lower than in the femoral neck or lumbar spine.[46,47] There is also evidence for pleiotropic effects, in which BMD in various skeletal sites is determined by both common and site-specific sets of genes.[36,48] Genetic factors have been shown to contribute significantly to the interindividual variance of bone formation markers (both osteocalcin and collagen C-terminal propeptide of type 1 collagen) in premenopausal twins.[33,34,48]

The involvement of multiple genetic and nongenetic factors suggests that osteoporosis is indeed a complex phenotype. As for many other multifactorial diseases, osteoporosis is also determined by environmental factors, and possibly by the interaction between environmental and genetic factors. Genetic variations do not necessarily cause osteoporosis or fracture, but rather influence an individual's susceptibility to specific environmental factors and so modify the disease risk. The dissection of gene–environmental interaction effects on bone phenotypes is still a major challenge in genetic research.

2.1 Candidate Genes

During the past 2 decades, intensive searches for specific genes linked to osteoporosis phenotypes have been attempted with significant success. Gene-search studies have been based on the two major approaches, initially candidate gene and more recently genome-wide studies.[49] The candidate gene approach has been used within the context of association studies, in which specific DNA variants are analyzed in cases and unrelated controls. This straightforward design has been used extensively in the field of osteoporosis, but it suffers from a number of shortcomings. The selection of appropriate controls can be a challenge, particularly for fractures that occur mainly in later life. The arbitrary classification of BMD into a dichotomous variable (e.g., osteoporosis vs. nonosteoporosis) does not exclude the possibility that a nonosteoporotic individual will develop "osteoporosis" in the future. Any statistically significant association between a specific gene variant and fracture may not necessarily indicate a causative relationship, because such an association can have arisen from population stratification and, even when a gene is associated with the fracture outcome, it may not be the gene studied but due to linkage disequilibrium with a causative gene that can be considerably distant along the chromosome.[50]

Nevertheless, based on this commonly used approach, several gene polymorphisms (including vitamin D receptor, collagen type Iα1, osteocalcin, IL-1 receptor antagonist, calcium-sensing receptor, α2HS glycoprotein, osteopontin, osteonectin, estrogen receptor α, interleukin-6, calcitonin receptor, collagen type Iα2, parathyroid hormone, and transforming growth factor α1 polymorphisms) have been proposed[51] (Table 9.1). However, the decade in which candidate gene association studies blossomed was also accompanied by frustration with conflicting findings and a lack of independent replication, mainly due to, among other factors, lack of statistical power[52] and/or to false positives.[53]

2.2 Genome-Wide Studies

Instead of focusing on a biologically plausible candidate gene, the genome-wide study scans the entire genome to identify chromosomal regions harboring genes likely to influence a trait. Two analytic strategies can be used in genome-wide study: linkage analysis and association analysis. Genome-wide studies, using either linkage or association analysis, are essentially a hypothesis-free approach, because they make no assumptions about the location and functional significance of associated loci or their products, only that there is some locus associated with a phenotype.[85] Genome-wide studies can examine the linkage or association between representative tagging-single-nucleotide polymorphisms (SNPs) spread throughout the genome and the susceptibility of diseases. As such, genome-wide studies can overcome weaknesses of the candidate gene design[86,87] by providing a holistic picture of genes that are likely to contribute to the susceptibility of disease. However, genome-wide studies have the major challenge of multiplicity of hypothesis tests. Since genome-wide

TABLE 9.1 Genes for Osteoporosis [Bone Mineral Density (BMD)] From Candidate Association Analyses

Genes	Gene names	Location	References
ARHGEF3	Rho guanine nucleotide exchange factor 3	3p14-p21	54
COLI1A1	Collagen type I alpha 1	17q21.33	55,56
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	15q21.1	57
DBP	D site of albumin promoter (albumin D-box) binding protein	19q13.3	58
ESR1	Estrogen receptor 1	6q25.1	59–61
ESR2	Estrogen receptor 2	14q	62
FNLB	Filamin B, β	3p14.3	63
FOXC2	Forkhead box C2	16q24.4	64,65
ITGA1	Integrin, alpha 1	5q11.2	66,67
LRP4	LDL receptor-related protein 4	11p11.2	68
LRP5	LDL receptor-related protein 5	11q13.4	66,69
MHC	Major histocompatibility complex	6p21	68,70
MTHFR	5,10-Methylenetetrahydrofolate reductase	1p36.3	71
PTH	Parathyroid hormone	11p15.3-p15.1	72,73
RHOA	Ras homologue gene family, member A	3p21.3	74
SFRP1	Secreted frizzled-related protein 1	8p12-p11.1	75
SOST	Sclerosteosis	17q11.2	66,76,77
SPP1	Secreted phosphoprotein 1 (osteopontin)	4q21-q25	66
TNFSF11	Tumor necrosis factor ligand superfamily, member 11 (RANKL)	13q14	66,78
TNFRSF11A	Tumor necrosis factor ligand superfamily, member 11a, NFk≡ activator (RANKL)	18q22.1	66,79
TNFRSF11B	Tumor necrosis factor ligand superfamily, member 11b (OPG)	8q24	66,80
VDR	Vitamin D receptor	12q13.11	81–83
WNT10B	Wingless-type MMTv integration site family, member 10B	12q13	84
ZBTB40	Zinc finger and BTB domain-containing protein 40	1p36	66

studies test the hypothesis of association for thousands of alleles, a statistically significant linkage or association is not necessarily a real phenomenon.[86]

Genome-wide linkage studies: Aim at identifying anonymous markers that cosegregate in families with multiply affected members. Linkage analysis is based on the principle that alleles at loci close together will tend to be inherited jointly; therefore, if a marker allele and a locus for a specific trait are close together on the same chromosome they are unlikely to be separated by crossovers during meiosis. A common metric of linkage is the log-of-the-odds (LOD) score, which measures the likelihood of linkage compared with no linkage. A linkage with a LOD score of >3.6 is considered significant at the genome-wide level, while a LOD score >2.2 is usually taken to indicate "suggestive" linkage.[88]

Linkage analysis has been successfully used to map causative genes linked to BMD and fracture. Genome-wide linkage analysis has identified several regions that are linked to variation in BMD (Table 9.2). Linkage analysis of data from a family with osteoporosis-pseudoglioma syndrome (OPPG), a disorder characterized by severely low bone mass and eye abnormalities, was able to localize the OPPG locus to chromosomal region 11q12-13.[89] At the same time, a genome-wide linkage analysis of an extended family with 22 members, among whom 12 had very high bone mass (HBM), suggested that the HBM locus also located within the 30 cM of the same locus.[90] In follow-up studies using the positional candidate approach, both research groups found that a gene encoding the low-density lipoprotein receptor-related protein 5 (LRP5) was linked to both OPPG and HBM.[91–93] The finding that the LRP5 gene is linked to

TABLE 9.2 Linkage Studies of Quantitative Trait Loci for BMD

Studies	Phenotype	Locus/marker	LOD score
Devoto et al.[95]	Hip BMD	1p36 (D1S540)	3.51
	Spinal BMD	2p23-24 (D2S149)	2.07
Niu et al.[96]	Forearm BMD	2p21 (D2S2141, D2S1400, D2S405)	2.15
	Distal forearm	13q34 (D12S788, D13S800)	1.67
Karasik et al.[97]	Hip BMD	6p21 (D6S2427)	2.93
	Spinal BMD	12q24 (D12S395)	2.08
	Trochanteric BMD	21qter (D21S1446)	2.34
Wilson et al.[98]	Spinal BMD	3p21	2.1–2.7
	Whole body BMD	1p36	2.4
Deng et al.[99]	Spinal BMD	4q32 (D4S413) 7p22 (D7S531) 12q24 (D12S1723)	2.12 2.28 2.17
	Wrist BMD	4q32 (D4S413)	2.53
Koller et al.[100]	Spinal BMD	1q21-23 (D1S484) 6p11-12 (D6S462) 11q12-13 (D11S987) 22q12-13 (D22S423)	3.11 1.94 1.97 2.13
	Hip BMD	5q33-35 (D5S422)	1.87

HBM was confirmed in a family study that included individuals with exceptionally high BMD but who were otherwise phenotypically normal.[92] This study showed that a missense mutation (G171V) was found in individuals with high BMD.[93] Another family-based study identified 6 novel mutations in the LRP5 gene among 13 confirmed polymorphisms that were associated with different conditions characterized by increased BMD.[94] The conditions included endosteal hyperostosis, van Buchem disease, autosomal dominant osteosclerosis, and osteopetrosis type I. The discovery of the LRP5 gene has opened up a new chapter of research in the genetics of osteoporosis.

Genome-wide association studies (GWAS): Are a powerful approach for searching for genes that are associated with a trait. GWAS can be seen as a hypothesis-free approach that scans hundreds of thousands of common SNPs (minor allele frequency usually greater than 5%) in the entire genome for single SNP associations in an unbiased manner. The basic idea is to test for differences in allelic frequency of anonymous genetic variants between cases and controls, unadjusted for any current understanding of disease etiology. The underlying idea of GWAS is linkage disequilibrium (i.e., association between genetic variants). That is, identified variants may be associated with other variants of potential interest more frequently than expected by chance. The SNP variants may also lie many hundreds of kilobases upstream or downstream from a known putative gene.

Statistically, in the presence of hundreds of thousands of tests, there is the real possibility of false-positive finding in GWAS. Just as with the evaluation of a diagnostic test, where one needs to know specificity, sensitivity, and positive predictive value, the reliability of a statistical association can also be evaluated by three analogous parameters: the observed *P* value, the observed power (sensitivity) given an effect size, and the prior probability of a true association.[101] The *P* value may be viewed as equivalent to the false-positive rate of a diagnostic test; it is the probability of observing the current data (or more extreme data) given that there is no true association. Power is the probability that a study will identify a true association if it exists, which is equivalent to the concept of sensitivity of a diagnostic test. Prior probability is a subjective probability of a true association. Based on these three parameters and by using the Bayesian approach, it is possible to determine the probability of no true association given a statistically significant finding or the false-positive report probability (FPRP).[102] Of the three parameters for evaluating FPRP, the prior probability is the most difficult parameter for which to assign a weight. This probability is dependent on the number of gene variants that affect fracture

susceptibility, which is unknown. Indeed, we do not know how many genes are involved in the regulation of, or are relevant to, the underlying susceptibility to osteoporotic fracture. However, we do know that in the human genome, there are about 3 billion base pairs,[103] and that on average, more than 90% of the differences between any two individuals is due to common variants where both alleles are present in at least 1% of the population.[104] Therefore, it has been hypothesized that the susceptibility to common diseases, such as osteoporosis is caused by relatively few common genetic variants with low effect size (i.e., the "common gene–common variant" hypothesis).[105] Under this hypothesis, it has been estimated that the number of genetic variants that are associated with a common disease is about 100 or less.[106] It has also been estimated that the number of common variants in the human population is about 10 million.[107] Therefore, it may be reasonable to assume that the probability that a randomly selected common variant is associated with the risk of fracture is 1/100,000 or 0.000001. Based on these assumptions, it has been suggested that a claim of association from a GWAS can be made if P value (less than 5×10^{-5})[108] or more stringently, 5×10^{-8}.[109]

Recent GWAS have identified many susceptibility loci for BMD and fracture risk. The earliest GWAS study in osteoporosis examined the association between 71K genetic variants and BMD measured at different skeleton sites, and found evidence of association for 40 SNPs. Although the study was then considered to be underpowered, several SNPs identified in this study were located in potential osteoporosis-associated genes, such as *MTHFR*, *ESR1*, *LRP5*, vitamin D receptor *(VDR*), and *COL1A1* genes.[110] Another GWAS screened 300K variants in an Icelandic population, and found that variants in the *ZBTB40*, *ESR1*, *OPG*, *RANKL* genes, and those in a novel region 6p21 were significantly associated with BMD at genome-wide threshold ($P < 5\times10^{-8}$).[70] This study also suggested some loci associated with fracture risk, including variants in the 1p36, 2p16, *OPG*, *MHC*, *LRP4*, and *RANK.* In the meantime, a GWAS in United Kingdom and Rotterdam cohorts found that variants in the *TNFRSF11B* and *LRP5* genes were associated with BMD, and the *LRP5* gene was also associated with fracture risk.[111]

Two metaanalyses of GWAS showed that variants in the *ZBTB40*, *ESR1*, *LRP4*, *LRP5*, *TNFSF11*, *SOST,* and *TNFRSF11A* genes were associated with BMD,[66,112] and that variants in the *LRP5*, *SOST*, and *TNFSF11A* were associated with fracture risk.[66] The latest metaanalysis involving 81,949 cases and 102,444 controls, identified 56 loci that are associated with BMD and 13 of these 56 loci were associated with fracture.[113] Overall, 60 loci have been found to be associated with BMD, and 15 loci associated with fracture.[114]

Whole genome sequencing (WGS): The search for genes associated with osteoporosis has been driven by two competing hypotheses: common disease–common variant hypothesis and common disease–rare variant hypothesis. The common disease–common variant hypothesis postulates that the susceptibility to common disease is determined by multiple common genetic variants (e.g., more than 5% minor allele frequency), each with low penetrance (or low magnitude of association). On the other hand, the common disease–rare variant argues that the susceptibility to disease is caused by multiple rare variants, each with high penetrance. The common disease–common variant hypothesis really underlies the GWAS approach. In recent years, remarkable progress in genome-sequencing technologies has facilitated a more thorough examination of exonic regions of the chromosome, which code for functional proteins, to identify potentially causative genes. Sequencing determines every nucleotide in a DNA sequence, including rare mutations that GWAS cannot identify. Therefore, in theory, these approaches, termed whole exome sequencing and WGS, are more likely to identify rare genetic variants that have a greater effect size. It has been proposed that between 80% and 90% of inherited disease-causing mutations are located within protein coding regions. If this was the case then, by analyzing just 1% of the genome using whole exome sequencing, it would be possible to identify almost 90% of disease-causing mutations.

Using WGS approach with extensive data from the UK10 Project, a genetic variant near the EN1 gene was discovered to have strong effect on BMD and fracture risk. This variant (rs11692564, located in noncoding regions) has a minor allele frequency of 1.6%, but the allele was associated with 0.2 SD higher lumbar spine BMD, and a 15% lower odds of fracture.[115]

Another successful application of WGS is the recognition of variants of the LGR4 gene associated with BMD and fracture risk. LGR4 is a member of the G protein-coupled receptor (GPCR) superfamily, which has various roles in development. Using WGS approach, a rare nonsense mutation in the LGR4 gene was found to be associated with BMD and fracture risk[116]; as previously identified in GWAS.[68] A recent study also making use of the WGS technology found two rare missense mutations (p.Gly496Ala and p.Gly703Ser) in the COLIA2 gene, a gene that is known to be associated with osteogenesis imperfecta.[117] Both variants were associated with 0.5–0.9 SD lower BMD and increased fracture risk (odds ratio = 4.4 for p.Gly496Ala and 7.0 for p.Gly703Ser).

2.3 Pathways

The genes identified by GWAS and WGS so far are involved in four main pathways: the RANK-RANKL-OPG

pathway (*TNFRSF11B*, *TNFRSF11A*, and *TNFSF11* genes); the Wnt-β-catenin pathway (*LRP5*, *LRP4,* and *SOST* genes); the estrogen endocrine pathway (*ESR1* gene); and the 1p36 region (*ZBTB40* gene) (Table 9.3).

The RANK-RANKL-OPG system was independently discovered by four groups in the 1990s,[128–131] and has since led to major advances in the understanding of the pathogenesis of osteoporosis. The system is involved in the regulation of bone modeling and remodeling, such that RANK/RANKL signaling controls the formation of osteoclasts from their precursors, whereas OPG by binding to RANKL protects the bone from excessive bone resorption.[132] The genes in the RANK-RANKL-OPG pathway include *TNFRSF11A* and *TNFSF11* genes encoding RANK and RANKL, respectively. The genes are members of the TNF superfamily. Binding of RANKL to RANK stimulates the formation and differentiation of osteoclasts, which in turn increase bone resorption.[133] OPG is an inhibitor of this pathway that binds to RANKL and prevents the interaction between RANKL and RANK. Genetic variants within the *TNFRSF11A* gene[134] and *TNFRSF11*[134] and *OPG*[79,134] were found to be associated with BMD and fracture in both candidate gene association studies and GWAS.[68,70]

The Wnt-β-catenin signaling pathway: Wnts are secreted glycoproteins that bind to receptors of the Frizzled and LRP families on the cell surface. Wnt proteins constitute a family of secreted signaling molecules that control cell–cell interactions during development. The Wnt signal is transduced to β-catenin, which enters the nucleus and forms a complex with TCF to activate transcription

TABLE 9.3 Some Key BMD-Associated Genes From Genome-Wide Association Studies (GWAS)

Genes	Gene names	Location and SNP	References
ADAMTS18	A disintegrin and metalloproteinase with thrombospondin motifs 18	16q23; Rs16945612	118
ALDH7A1	Aldehyde dehydrogenase 7 family	5q31; Rs13182402	119
CTNNB1	Catenin (cadherin-associated protein), beta 1	3p21; Rs7935346	70
CRHR1	Corticotropin releasing hormone receptor 1	17q12-q22	112
DCDC5	Doublecortin domain containing 5	11p14.1	112
FAM3C	Interleukin-like EMT inducer; predicted osteoblast protein	7q31; Rs7776725	120
FLJ42280	Putative uncharacterized protein FLJ42280	7q21.3	112
GPR177	Wntless homolog (*Drosophila*)	Chr 3; Rs2306033	70
IL21R	IL-21 receptor	16p11; Rs8057551 Rs8061992 Rs7199138	121
JAG1	Protein jagged-1	20p12.1-p11.23; Rs2273061	122
MARK3	Microtubule affinity-regulating kinase 3	Chr 12; Rs2010281	70,112
MEF2C	Myocyte enhancer factor 2C	13 45.0 cM	112
MHC	Major histocompatibility complex	6p21; Rs3130340	70
OSBPL1A	Oxysterol binding protein-like 1A	18q11.1; Rs7227401	123
PLCL1	Phospholipase C-like 1	2q33; Rs2278729	124
RAP1A	RAP1A, member of RAS oncogene family	1p13.3; Rs494453	123
RTP3	Receptor (chemosensory) transporter protein 3	3p21.3; Rs7430431	125
SFRP4	Secreted frizzled-related protein 4	7p14.1; Rs1721400	120
SOX6	SRY-box containing gene 6; transcription factor SOX-6	11p15.3; Rs297325, Rs4756846	126
SP7	Transcription factor 7	Chr 15	68
STARD3NL	STARD3 N-terminal like	7p14-p13	112
TBC1D8	TBC1 domain family, member 8	2q11.2; Rs2278729	123
TGFBR3	TGF-beta receptor type 3	1p33-p32; Rs7524102	68,70,126,127

of Wnt target genes.[135] Mutations in Wnt-signaling pathway result in birth defects, cancer, and other diseases. In bone metabolism, the Wnt pathway contributes to the process of bone formation by regulating the differentiation and proliferation of osteoblasts, and bone mineralization. Expression of the *LRP5* gene, which is an element of this pathway, signals into the nucleus to activate bone formation. The *LRP5* gene was identified as a candidate gene of BMD[136] and fracture risk.[111,137] Although several polymorphisms in the *LRP5* gene have been studied, variants in exon 9 (V776M) and exon 18 (A1330V) are the two most widely investigated.[138] An inhibitor of the Wnt-pathway, SOST, was also a candidate gene of osteoporosis. Expression of *SOST* was found to inhibit bone formation by preventing the binding of Wnt to LRP5.[139] Polymorphisms of the *SOST* gene were associated with BMD variation in both a candidate gene study[76] and GWAS.[68]

The estrogen pathway: The estrogen endocrine pathway has long been known to play a key role in the formation and maintenance of bone mass.[140] The *ESR1* gene has been shown to be a candidate gene for the genetic effect on BMD[141] and fracture risk[141–143] in several populations. Moreover, polymorphisms of the *ESR1* gene were found to be associated with bone loss.[144] Although the mechanism of effect of these genetic variants on BMD is unclear, intronic variants in this gene are thought to exert an effect on the efficiency of gene transcription.[145]

The 1p36 region: The chromosomal region 1p36 has been implicated in the regulation of bone mass by linkage analysis.[95,146] The *ZBTB40* gene situated in this region was identified as a candidate gene for BMD variation in some recent GWAS[68,70] and in metaanalysis.[112] However, any function of the *ZBTB40* in bone homeostasis is still largely unknown.

Apart from the aforementioned pathways, genes in the endochondral ossification pathway have also been shown to be associated with BMD or fracture. Endochondral ossification is a major embryonic process of bone formation,[147] in which osteoblasts deposit collagen and noncollagenous proteins on a cartilaginous template, which subsequently mineralizes. GWAS have identified several genes in this pathway, including genes involved in the development of cartilage, cartilage ossification, and osteoblast differentiation: *IBSP, PTHLH, RUNX2, SOX6, SOX9, SPP1*, and *SP7*.

3 GENE–GENE INTERACTION AND "MISSING HERITABILITY"

Genetic variants identified so far from candidate gene and GWAS studies explain only a modest proportion (less than 3%) of the variance in fracture liability[112] raising the question of "missing heritability." This "missing heritability"[148,149] has also been observed in other heritable conditions, such as cancer[150] and cardiovascular diseases.[151]

In most genetic studies of fracture, the association between putative loci and fracture risk has been analyzed in a one-by-one SNP strategy, where the effect of each SNP on fracture risk is assumed to be independent from the effect of other SNPs. If a SNP itself is sufficient to confer an increased risk of fracture, it can be detected by comparing the frequency of the variant in fracture and nonfracture group. However, if a SNP confers an increased risk of fracture only in the presence of other SNPs, then they can only be detected when these variants are analyzed simultaneously by modeling their gene–gene interactions. At the individual level, gene–gene interaction predicts that two individuals can have different fracture risk even if they share the same genotype at one locus. At the population level, epistasis suggests that heterogeneity and incomplete penetrance of fracture is expected. Therefore, the "missing heritability" may be explained by the presence of gene–gene interaction effects that have not been taken into account in previous studies.

Gene–gene interaction is defined as the effect of a genetic variant being modulated by a variant or variants of other genes on the same or different chromosomes.[152] Gene–gene interaction can result in differences in genetic penetrance, and mask the effect of putative genes. Thus, a SNP may not be associated with a phenotype in a single analysis, but it is associated with the phenotype in the presence or absence of another SNP. Although gene–gene interaction is recognized as a ubiquitous phenomenon in the genome,[153] it is difficult to detect the effect due to the lack of powerful methodology and generally insufficient sample size.[154] Current linear statistical genetic methods used for analyzing and detecting gene—phenotype association in human populations are not sensitive enough to detect nonlinear interacting effects due to the combinatorial complexity of gene–gene and gene–environment interactions. The complexity is even more formidable if one considers data from multiple genes (rather than two genes), with higher dimensional values leading to very large number of comparisons, resulting in an increase in type I errors. In linkage scans in humans, testing 400 markers for pairwise epistasis involves testing 79,800 combinations. In GWAS, more than 10^{11} two-marker combinations may need to be studied; for three-marker interactions more than 10^{16} possible combinations are involved. As linkage studies in common human diseases in particular have low power to identify the genetic effects from single loci, it is easy to understand why the power to detect epistasis is very low, and why few reported interactions have been confirmed in subsequent studies.

4 PHARMACOGENETICS OF THERAPEUTIC RESPONSE

During the past 2 decades, several major advances in the treatment of osteoporosis have been made with many therapeutic options available. Antifracture therapies can be broadly divided into two groups: antiresorptive and bone-forming (anabolic) agents.[26] The former decreases bone turnover, while the latter increases bone formation. The antiresorptive agents include bisphosphonates (e.g., alendronate, risedronate, clodronate, etidronate, ibandronate, zoledronic acid, raloxifene, and calcitonin), while anabolic agents include teriparatide (recombinant human parathyroid hormone)[1–34] and possibly strontium ranelate. Both types of agents have been shown to reduce fracture risk for some, although not necessarily all, fragility fractures.[155] In most clinical trials, the relative risk reduction in fracture incidence has been 25%–60% with significant variability in response across individuals and across fracture sites.

The variation in individual clinical response to osteoporosis treatment is also present in BMD. Indeed, the SD of change in BMD induced by antiresorptive drugs is up to twice the mean rate of change. As a result, while the majority of patients experience an increase in (or stabilization of) BMD, a small proportion (perhaps up to 10%) of patients apparently still lose bone.[156] Thus, although few patients experience absolutely no therapeutic effects following typical antiresorptive treatment, no treatment completely prevents bone loss and none prevents all fractures.

While patients' characteristics, such as age, gender, ethnicity, and concomitant disease may affect drug response, genetic factors may also determine an individual's response to pharmacological therapy for each specific drug.[157] Indeed, there is considerable evidence suggesting that genetic factors affect the variability in drug response.[158] For example, in studies of twins, identical twins were more similar than nonidentical twins with regard to the plasma half-life of numerous drugs, providing the best experimental indication of strong genetic components in drug elimination.[159] Genetic information can, thus, potentially be used to identify patients who are likely to respond (or not respond) to pharmacological therapy.[160]

Some drugs used in osteoporosis therapy, bisphosphonates, for example, are not subject to metabolism, but absorption, which is poor for oral bisphosphonates could be affected. Other therapies are metabolized to active components or as part of their elimination pathways. During the past 2 decades, there have been at least 20 studies on the association between genetic polymorphisms and response to antiresorptive therapies. Almost all studies have focused on genes identified from the candidate gene association analysis (e.g., VDR, COLIA1, LRP5), and used BMD change as an endpoint. All these studies were based on small sample sizes, thus "significant" associations observed may not be good evidence for a true association. On the other hand, a statistically nonsignificant association is more likely inconclusive rather than reliable evidence of no association. Some key findings of pharmacogenetic studies are summarized in Table 9.4, and can be highlighted as follows:

Response to hormone replacement therapy (HRT): HRT has been used in the treatment of osteoporotic patients, and can effectively increase BMD[173] and reduce fracture risk. Results of a metaanalysis of randomized controlled trials (RCT) suggested that postmenopausal women on HRT had lower risk of nonvertebral fracture (relative risk: 0.74; 95% confidence interval: 0.56–0.94), and the effect was particularly pronounced in women younger than 60 years.[174] However, it was estimated that 8% of women on HRT were nonresponders.[175]

Recent studies have attempted to elucidate the differential effects of HRT on BMD, and results suggested that polymorphisms of the estrogen receptor alpha (ERα), VDR, and MTHFR genes might be involved in the determination of responders versus nonresponders. Short TA repeat alleles at the promoter of the ERα gene (rs3138774) have been reported to be associated with reduced BMD and increased risk of fracture.[142,176] A study on 79 postmenopausal women on HRT for 3 years, carriers of *PP* and *XX* genotypes of the ER gene responded with higher increases in BMD at the lumbar spine and femoral neck.[177] This finding had also been previously noted in a Thai study on 124 women, among whom those with the *P* allele had greater increases in BMD.[178] Furthermore, among postmenopausal women on equine estrogen alone or in combination with medroxyprogesterone, those with short TA alleles responded with greater increases in BMD at the lumbar spine.[179] However, this interesting finding was not replicated by others.[144,180–186]

A series of studies by Palomba and coworkers[134–136] suggested that among postmenopausal women who were on HRT treatment, the *b* allele of the VDR Bsm-I polymorphisms was associated with a greater increase in BMD than those carriers of the *B* allele. Moreover, Deng[127] and Kurabayashi[129] observed that postmenopausal women treated with equine estrogen for at least 3 years, women with TT genotype (which is in high linkage disequilibrium with the BsmI bb genotype) responded with greater increases in lumbar spine BMD. There was no significant effect of Sp1 genotypes within the COLIA1 gene on BMD changes among women treated with estradiol.[187]

Response to selective estrogen receptor modulators (SERMs): SERMs are FDA-approved for the treatment

TABLE 9.4 Studies on Association Between Candidate Genes and Response to Treatment

Genes	Drug studied	Phenotype	Main findings
VDR	Raloxifene	BMD changes	GG genotype was associated with higher increase in lumbar spine BMD[161]
VDR	Etidronate	BMD changes	GG genotype was associated with higher increase in lumbar spine BMD[162]
VDR	Alendronate + HRT, Alendronate + Raloxifene, Alendronate or Teriparatide	BMD changes	Patients with AG and GG genotypes had greater increase in BMD[163,164]
ER	Raloxifene	BMD changes	Patients with PvuII PP genotype were associated with higher increase in BMD[165]
ER	Etidronate	BMD changes	No significant association[166]
COLIA1	Etidronate	BMD changes	GG genotype was associated with greater increase in femoral neck BMD[167]
COLIA1	HRT	BMD changes	GG genotype was associated with greater increase in femoral neck BMD[168]
FDPS	Alendronate or Ibandronate	BMD changes	Patients homozygous CC genotype for rs2297480 had a decreased response of bone turnover markers[169]
FDPS	Alendronate or Risedronate	BMD changes	Suggestive association between GGPS1 −8188A ins/del polymorphism and femoral neck change in Korean women[170]
LRP5	Risedronate	BMD changes	No statistical association between polymorphism within the gene and response to treatment[171]
OPG	Alendronate	BMD changes	BMD change at the intertrocher was higher in women with AA genotype, but at site T245G, the change was higher in women with the TT genotype[172]

of osteoporosis, with a significant efficacy.[188] Among patients on raloxifene, carriage of the *B* allele (of the VDR) was associated with a greater increase in BMD than the *b* allele carriers.[161] Among those on combined alendronate and raloxifene there was no significant association between VDR polymorphisms and BMD change. These results illustrate the potential for interaction between VDR polymorphisms and various antiresorptive drug therapies in BMD change.[164]

Response to bisphosphonates: Oral bisphosphonates are considered first-line treatment for osteoporotic patients. RCT have consistently shown that bisphosphonates can increase BMD or prevent bone loss and reduce fracture risk,[189,190] including hip fracture risk.[191] However, as in the case of estrogen and HRT, not all patients respond to bisphosphonate therapy, and it is hypothesized that genetic factors partially account for the variation between responders and nonresponders. Studies by Palomba and coworkers[161,163,164] suggested that among postmenopausal women on alendronate, carriers of the *b* allele of the VDR Bsm-I polymorphisms had greater increase in BMD than those carriers of the *B* allele.

The response to bisphosphonate therapy may also be associated with the collagen I alpha 1 (COLIA1) gene which has been shown to be associated with variation in BMD and fracture risk.[192] In a study on 108 perimenopausal women with osteopenia who were randomized to receive cyclical etidronate (a bisphosphonate) or placebo, femoral neck BMD increased in carriers of the Sp1 (dbSNP rs1800012) SS genotype (~64% in the population) but decreased in those carrying the *s* allele (Ss and ss genotypes).[167] It should be noted that this Sp1 polymorphism has also been reported to be associated with the response to human growth hormone in men and women with growth hormone deficiency:[193] Individuals with the SS genotype required a higher subcutaneous dose than those with the ss genotype.[193] However, because these studies were based on relatively small sample sizes and the confidence intervals of effect size were wide, no definite conclusion on the association between the Sp1 polymorphism and drug response can be reliably inferred.

There is evidence that genetic variants in the *VDR* gene influence intestinal absorption of calcium leading to an effect on bone mass.[194,195] In a longitudinal study of calcium supplementation in the elderly, participants with *VDR Bsm*I variant carrying BB genotype had a higher proportion of bone loss at lumbar spine than those with Bb and bb genotypes.[196,197] Women with BB genotype were also found to have reduced efficiency of calcium absorption on a regimen of low calcium intake.[198] Looked at another way, these results imply that people with BB genotype might be more likely to respond to a calcium-rich diet.

Osteonecrosis of the jaw (ONJ): Apart from antifracture efficacy, bisphosphonates are commonly used in the management of patients with advanced cancers that have metastasized to the bone, who are at high risk of bone pain and fractures. Several cancers can involve or metastasize to the bone, including lung, breast, prostate, multiple myeloma, and others. In cancer chemotherapy, bisphosphonates are given intravenously, and usually in more frequent and much higher cumulative doses than used in osteoporosis treatment. In these patients with cancers, there is a risk of ONJ. The risk of ONJ in osteoporosis treatment is much lower than in the cancer therapy situation and arguably even if it occurs is much less clinically severe. During the past 20 years, there have been 368 cases of ONJ reported to be associated with bisphosphonates treatment; among whom ~95% occurred in patients with myeloma or breast cancer.[199] In a study on more than 260,000 patients with cancers of the breast, lung, and prostate and who had been on bisphosphonates, the investigators found 224 ONJ cases; an incidence of 0.3%.[200]

The risk of bisphosphonates-related ONJ is associated with genetic factors. In a genome-wide association study on 22 cases of ONJ and 65 age-matched controls, by screening >500,000 SNPs, the investigators reported 4 SNPs (rs1934951, rs1934980, rs1341162, and rs17110453) mapped within the Cytochrome P450-2C gene (CYP2C8) to be associated with the risk of ONJ.[201] The relative risk of ONJ associated with each of the SNPs ranged between 10 and 13.[201] However, in a subsequent study,[202] the association between rs1934951 and ONJ risk was not confirmed. In another recent GWAS study on 30 cases of ONJ and 60 genetically matched controls, it was found that the SNP rs17024608 within the RBMS3 gene was associated with ONJ risk ($P < 7 \times 10^{-8}$). Individuals positive for the SNP rs17024608 were almost 6 times more likely to develop bisphosphonates-induced ONJ.[202] However, further studies using case control and cohort design found no clear association between the risk of ONJ and variants in the RBMS3, as well as IGFBP7, ABCC4 genes,[203] CYP2C8,[201] COLIA1, RANK, MMP, OPG,[204] FDPS,[205] or MMP2 gene [206] (Table 9.5).

In summary, most studies of genetics of therapeutic response have been largely based on the candidate gene approach, with limited sample size. Experience in the candidate gene association studies of fracture suggest that this type of design has often yielded conflicting findings and poor replication, mainly due to lack of statistical power[52] and false positive.[207] Therefore, all of the reviewed findings are somewhat mixed, and yet to be replicated in independent samples. Moreover, cost-effectiveness studies are needed to optimize the selection of therapies.

TABLE 9.5 Summary of Findings From Genetic Analysis of Bisphosphonates-Induced Osteonecrosis of the Jaw (ONJ)

Author	Design and participants	Genes	Main findings
Sarasquete et al.[201]	Case-control; multiple myeloma, 22 cases and 65 matched controls	CYP2C8	Three SNPs within the CYP2C8 gene (rs 1934951, rs1934980, rs1341162, and rs17110453) associated with increased risk of ONJ
Nicoletti et al.[203]	35 cases and 18 controls	RBMS3, IGFBP7, and ABCC4	Suggestive association
Such et al.[202]	Case-control; multiple myeloma, 9 cases, and 79 matched controls	CYP2C8	No statistically association between SNP rs1934951 and ONJ
Katz et al.[204]	Cohort study, patients with multiple myeloma on intravenous bisphosphonate	COLIA1, RANK, MMP, OPG, OPN	Trend of statistical significance
Marini et al.[205]	Case control study. 68 patients with multiple myeloma or metastatic cancers receiving bisphosphonates	FDPS	SNP rs2297480 significantly associated with risk of ONJ
Lehrer et al.[206]	Case series, 7 patients with ONJ and were on bisphosphonates.	MMP2	No data, only hypothesis of association

5 TOWARD INDIVIDUALIZED ASSESSMENT AND INDIVIDUALIZED TREATMENT DECISIONS

Osteoporosis research is entering an exciting phase of translation of highly promising research findings into clinical applications. Within the translational domain, models for predicting individuals at high risk of fracture have been developed. Given the availability of multiple treatments and their associated, albeit uncommon, adverse events, there is also clearly a pressing need for research to guide treatment decisions and predict patient responses to drugs. Pharmacogenetics and pharmacogenomics can be applied to address these needs.

Using established clinical risk factors, a number of predictive models have recently been developed and implemented.[208–210] The predictive accuracy of these models have been less than perfect, with the area under the receiver operating characteristic curve ranging between 0.70 and 0.80.[209,210] Most predictive models have low sensitivity and high specificity. Thus, there is room for further improvement of prognostic accuracy of the current models by incorporating genetic markers.

There are potentially major advantages of using genetic markers as a prognostic factor of fracture risk. First, since an individual's genotype is time-invariant, it is easier to estimate its effect size and to incorporate its information into a prognostic model. Furthermore, the time-invariant nature of genotypes can be used in the prediction of fracture at a younger age well before the conventional risk factors, such as BMD become informative. Second, as the association between a genetic variant and fracture risk appears to be independent of clinical risk factors, the use of such a genetic marker can potentially improve predictive value. Third, although there is no "genetic" therapy for individuals at high risk of fracture, the use of genetic markers could help segregate individuals at high risk from those with low risk of fracture, and thus help manage the burden of osteoporosis in the community.

The translation of genetic research into clinical applications faces challenges, particularly in relation to the effect size of genetic variants. Two common features of the genes identified so far are that their allelic frequency in the general population is highly variable (ranging from 1% to 61%), and the effect size is very small, with relative risk ranging between 1.05 and 1.5. In the presence of such small effect sizes, it can be anticipated that the contribution of any single genetic variant to fracture prediction is minimal. Even for a gene conferring a relative risk of 3, the AUC attributable to this gene is barely 0.51 (in the absence of clinical risk factors). Even with five genes, each conferring a relative risk of between 2 and 3, the AUC is still 0.60, which is not useful for predicting fracture. These findings are in part driven by the fact that genes variants of larger effect size are inherently (mathematically) more rare. This finding suggests that the contribution of any single gene to fracture prediction, no matter how large the effect size, is likely limited and may be of limited use in the clinical setting. However these analyses do not exclude the likely combination of common gene variants of small effect size with less common variants of larger effects size. Since the currently identified risk-associated allele of the genes related to fracture risk have low penetrance, the combination of multiple genes could be used to develop clinically useful tests. Our recent work suggests that the integration of genetic profiling, either in the form of a genetic risk score or individual genes, into the current prognostic models could significantly improve the predictive accuracy of fracture risk for an individual. Our recent simulation study suggested that a profile of up to 25 genes (each with relative risk of 1.1 to 1.35 and gene frequency ranging from 0.25 to 0.60) in the presence of clinical risk factors—with or without BMD—could achieve an AUC of 0.80, indicative of clinical usefulness.[211] Until now, very few genes have been implicated in the determination of fracture risk. A recent metaanalysis of 150 SNPs found that only 5 SNPs from 4 genes were consistently associated with fracture risk with RR ranging from 1.1 to 1.4.[66] Although a single gene may not help, a genetic profile of multiple genetic variants could help improve the assessment of fracture risk. Indeed, 2 empirical studies in postmenopausal women of Korean background reported that the genetic profile of 39 SNPs in 30 human genomic loci could increase the precision of nonvertebral fracture prediction and help to define the risk threshold[212] while 35 risk alleles were significantly associated with the risk of vertebral fracture[212,213] in patients on bisphosphonate. Taken together, these results suggest that genetic profiling may be useful in the identification of high-risk individuals.

The aim of individualized prognosis is to provide an accurate and reliable prognosis of fracture for an individual, and to help improve the management of the individual's predisposition to fracture. At present, individuals with low BMD (i.e., *T*-scores less than −2.5) and/or with a history of prior low trauma fracture are recommended for therapeutic intervention.[214,215] This recommendation is logical and appropriate, since these individuals have higher risk of fracture,[24,216] and treatment can reduce their risk of fracture.[191,217,218] However, because fracture is a multifactorial event, there is more than one way that an individual can attain the risk conferred by either low BMD or a prior fracture.[219] Moreover, each individual is a unique case, because no "average individual" exists in the population. The uniqueness of an individual can be defined in terms of the individual's environmental and genetic profile. Thus, the knowledge of genetics, in combination of clinical risk factors, could shift the current risk stratification (i.e., "one-size-fits-all") approaches to

a more individualized evaluation and treatment of osteoporosis.

An important application of pharmacogenetics is the optimization of treatment outcome and minimization of adverse effects from therapies. There is a paradox in clinical osteoporosis in that, while RCT are the gold standard for evaluating efficacy of a therapy in a population, they are suboptimal for evaluating efficacy for an individual. Indeed, results of RCT provide the best evidence for a group of individuals, but individual patients often differ in their response to drug therapies. Thus, there is reasonable uncertainty whether the drug that works for a group of individual is optimal for an individual. Moreover, while the variability in treatment efficacy is a reality, but there is also a large variability in adverse drug reactions associated with drug treatment. Therefore, the goal of pharmacogenetics and pharmacogenomics is to optimize the selection of therapy for an individual.

Recent pharmacogenetic studies have suggested a number of genes (e.g., VDR, COLIA1, ERα) that may be implicated in diverse physiological pathway, such as calcium metabolism and changes in BMD. However, these findings have poor replication, and consistent data remain rather elusive. These genes require further studies, as well as careful functional genomics working to identify any specific molecular events that may produce clinical effects in order to contribute to future drug development strategies. In osteoporosis, pharmacogenetic studies have focused on one major phenotype: changes in BMD. There is considerable variation in the phenotype, which reduces the power of small studies to distinguish real association from stochastic noise.

Finding genes that are associated with drug response is a challenging task. It is not clear how many genes are involved in the regulation of, or relevant to, the underlying drug response (e.g., efficacy and safety). Under the hypothesis that the variability in drug response is determined by multiple genes, each with low effect size, and given that the estimated number of common variants is ~10 million,[34] the probability of a randomly selected common variant being associated with drug response is very low, probably in the order of 1/100,000 or 0.000001. Even if there is a priori biologic justification, this probability may be around 0.001. Since the a priori is generally low, the probability of a true association between a genetic variant and drug response is also low. Using the Bayesian approach[102] and a prior probability of association set at 0.001 and 0.000001 (corresponding to that expected for a candidate gene and for a random SNP in a genome-wide association/analysis, respectively), most reported associations between the genetic variants and BMD changes had FPRP of greater than 0.20, which means that most of these associations are probably not "true." Genetic profiling, by combining the effects of many small effect loci, may be a better approach to define the association between genetic variation and response to antiosteoporotic therapies.

6 CONCLUSIONS

It is established that genetic factors play an important role in the regulation of BMD variation and fracture risk. There is now evidence that genetic factors may be involved in the determination of interindividual variation in response to osteoporosis therapies. It is highly likely that both fracture risk and drug response are modified by multiple genes (and perhaps interactions among genes), but it is not known how many genes are involved nor the mode of inheritance. In fact, with current methodology, it is unlikely that we will completely understand the causes of fracture, and why some individuals respond to drug therapies and others do not. However, it is possible to identify risk factors that identify a substantial proportion of individuals at risk and who would be amenable to intervention. Newly identified genetic variants in combination with clinical risk factors may help improve the accuracy of prognosis of fracture for an individual, and segregate individuals at high risk of fracture from those with lower risk and hence, lead to better management of the burden of osteoporosis in the general community.

This chapter has reviewed the polymorphisms or genes that have been shown to be associated with BMD, fracture risk, or response to therapies. In clinical practice, patients are highly heterogeneous with respect to risk profile, and the identification of genetic variants that contribute to the identification of high-risk individuals, as well as the response to therapies will be the next important step to replace the current general protocols with specific and individualized decision tools.

To date, no gene test has been developed for predicting the risk of fracture or the response to osteoporosis therapies. This is largely due to the lack of clarity about which genes are definitely associated with fracture risk or response to therapies. It is also not clear whether the two traits (e.g. fracture risk and drug response) have common or trait-specific genes. Although genetic tests for making treatment decision are not yet available, knowledge of the underlying mechanisms of adverse drug reactions will inform drug development process and enable better prediction of the potential toxicity of new drugs. Recent advances in genetic technologies, such as the sequencing of the entire human genome, the international HapMap project, and new genotyping technologies, and GWAS have created an opportunity to identify genes that are truly associated with fracture risk and drug response.[152] These molecular and bioinformatic approaches can potentially help guide future drug discovery and development, and enhance the possibility of personalized prevention and treatment of osteoporosis.

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CHAPTER

10

Genetic Testing and Counseling

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1 GENETIC TESTING

There are several ways that an individual's genome can vary from "normal." Specific molecular techniques have been developed to test for the different types of variants. Here we discuss the different types of variants associated with genetic disorders, and the types of genetic testing available now and in the future in molecular diagnostic laboratories.

1.1 Small-Scale Variants

Base-pair substitutions, the replacement of one nucleotide base by another, are the most abundant variant type in the human genome. Single-nucleotide polymorphisms (SNPs) are base-pair substitutions that are common in the population; by definition two or more versions of a sequence must be present in at least 1% of the general population to be classified as an SNP. SNPs make up about 90% of all human genetic variation, and occur every 100–300 bases along with the 3 billion base human genome. This variation in the DNA sequence can have an impact on how humans respond to disease, environmental factors, and therapies.[1] As of June 2016, the SNP database listed about 150 million SNPs in humans (NCBI SNP database build 148 for human, https://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi). The vast majority of SNPs are located in noncoding regions, including intergenic regions (stretches of DNA that contain few or no genes) and intronic regions (stretches of DNA within a gene that is removed by RNA splicing). This is not surprising given that although the haploid human genome contains about 3 billion base pairs,[2] only about 1.5% of the genome codes for proteins. The rest of the genome consists of noncoding RNA genes, regulatory sequences, introns, and noncoding DNA[3] and this is where most SNPs are found.

However, not all single-nucleotide changes are polymorphisms. Unlike SNPs, specific disease-causing variants are not common (occur in <1% of the population) and often occur within a gene's coding or regulatory regions, affecting the function of the protein encoded by the gene.[4] Single nucleotide variants, whether an SNP or a disease-causing variant, that are located in the coding regions (exons) can be further classified based on their effect on the amino acid sequence of the protein. Synonymous variants, also called "silent variants," do not change the amino acid, or protein product, because of the "degeneracy" of the genetic code. Although, in most cases, synonymous variants are not thought to have any consequences, there is increasing evidence that they can alter a phenotype due to effects on transcription, splicing, or translation.[5] Thus, the consequences of these variants may be hard to predict. Nonsynonymous variants change the amino acid coded for. When the nucleotide change results in the replacement of one amino acid for another it is called a missense variant; when the nucleotide change results in the gain of a stop codon it is called a nonsense variant. Sometimes a nucleotide change results in the loss of a stop codon (i.e., a stop codon changes to a codon that inserts an amino acid). Nonsynonymous variants that have a significant impact on protein structure may impact the phenotype.

Splicing variants are single nucleotide variants that occur in the regions of an intron that are required for removal of the introns and joining of the exons to form the

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final mRNA. Variants in the 3′ or 5′ invariant splice sites most commonly result in exon skipping, although they can also lead to activation of a cryptic splice site or intron retention.[6] Amino acids are deleted (exon skipping) or added (intron retention) to the protein. In addition, if the deleted or added DNA sequence is not a multiple of three, a frameshift occurs leading to a change in the amino acid sequence and very often a new stop codon downstream, truncating the protein. Clearly, splicing variants have significant impact on the protein and usually on the phenotype.

Indels are the insertion or deletion of one or several nucleotides within a sequence.[7–10] When the number of nucleotides inserted or deleted is a multiple of three, an in-frame insertion or deletion of one or more amino acids could occur, the reading frame downstream is not shifted, and the normal protein sequence resumes after the insertion or deletion. When the number of nucleotides is not a multiple of three, a frameshift occurs, the downstream amino acid sequence changes, and very often a new stop codon occurs downstream, truncating the protein. Thus, out-of-frame indels usually have a greater impact on protein structure and function than in-frame indels, and often impact the phenotype.

Repeat expansions occur in areas within the genome that contain trinucleotide repeating sequences; an increase, or expansion, in the number of these repeating sequences can occur in both coding and noncoding regions.[11] "Trinucleotide (or triplet) repeat disorders" are disorders that occur when there is an abnormal expansion of these DNA-triplets (trinucleotides). This type of insertion variant is known to be associated with a limited number of disease-causing genes, but constitutes an important category of variant and disease mechanism. Examples include Huntington disease and fragile X syndrome.[12]

Epigenetic modifications, finally, some disorders are caused by changes in the epigenetic modification pattern, such as DNA methylation.[13] There are regions in the human genome where the two alleles are differentially methylated and expressed depending on parent of origin (the imprinting phenomenon). Changes in the methylation pattern at these loci may disrupt the imprinting pattern and lead to imprinting disorders. Examples include Prader–Willi/Angelman syndrome (https://www.ncbi.nlm.nih.gov/books/NBK1330), as well as Albright hereditary osteodystrophy and pseudohypoparathyroidism.[14]

1.2 Large-Scale Variants

Large-scale variants affect at least one exon of a gene or a larger genomic segment. Copy number variants (CNVs) are imbalanced structure variants. Genomic DNA copy number gain or loss have been known for many years, but a renewed interest in CNVs is due to the finding that not only CNVs are much more abundant in the human genome than previously appreciated, but also they are now known to have a large impact on genome structure and function.[15] Furthermore, many CNVs, especially large ones, have significant clinical relevance. CNVs constitute the second most frequent variant type associated with genetic disorders.

Balanced structural variants, including chromosomal translocations and inversions, represent another type of large-scale variant. They can lead to disease if a gene is disrupted.

Another large-scale variant is referred to as copy neutral loss of heterozygosity (cn-LOH). LOH refers to the deletion of one allele of a gene. cn-LOH occurs when there is LOH but with no net change in the copy number. When both alleles originate from one parent, cn-LOH is referred to as uniparental disomy (UPD). In UPD, both copies of a chromosome, or part of a chromosome, are inherited from one parent and no copies from the other parent.[16] cn-LOH can also occur within a family due to identity by descent whereby family members inherit identical copies of the same ancestral allele. Identity by descent may be due to distant relatedness between two individuals or consanguinity indicating recent relatedness.[17]

Traditionally, microsatellite marker based genotyping methods have been used to detect UPD. Recent microarray platforms are able to detect large segments of cn-LOH and UPD in a genome-wide manner.

1.3 Evolving Approaches to DNA-Based Genetic Testing

The most-effective method for detecting most small-scale variants has been Sanger sequencing of DNA fragments amplified using the polymerase chain reaction (PCR).[18] To assess the number of repeats in the case of repeat expansions, PCR-based assays, and occasionally Southern-blot based assays, are necessary.

A fairly common technique used in a variety of circumstances is multiplex ligation dependent probe amplification (MLPA).[19] MLPA is a form of multiplex PCR in which multiple genomic targets are amplified using a common primer pair located at the outside ends of the multiple oligonucleotide probe pairs specific to the genomic targets. Thus, the strength of amplification signal reflects the amount of genomic target (inferred as copy number). Methylation-specific MLPA can be used to assess the methylation status of disease-related genes in the case of epigenetic variants. In methylation-specific MLPA specific probes are selected at the methylation loci and the genomic DNA is treated with methylation sensitive restriction enzymes that disrupt the methylated DNA preventing the ligation, hence reducing the amplification signal. The strength of the amplification signal at the methylated sites compared to the signal generated from DNA that has not been treated with enzyme reflects

the methylation level. MLPA is used to detect variants and gene deletions/duplications. In fact, for disorders in which deletions or duplications are common, MLPA is performed first as it is much easier and faster than sequencing. MLPA is also used to complement Sanger sequencing, in which case Sanger sequencing is performed first and, if the result is normal, MLPA is then performed to detect possible exonic deletions; sometimes, the two tests are performed simultaneously.

For more than 10 years now, microarray-based genomic profiling technologies have enabled the effective analysis of large variants, which cannot be reliably detected by conventional PCR and Sanger sequencing-based assays, with much improved sensitivity, resolution, and reproducibility. There are two basic microarray-based genomic profiling types.[20] Comparative genomic hybridization (CGH) detects copy number changes (unbalanced rearrangements). DNA from a patient and a normal control are labeled with different fluorescent tags, mixed, and then hybridized to normal chromosomes. Differences in the ratio of fluorescent labeling with the patient versus the control DNA can identify abnormal regions in the patient's genome. Currently, oligo-based CGH arrays are most often used and offer a resolution of 50 kBp. The subsequent addition of SNP probes onto CGH arrays have enabled detection of both CNV and LOH by CGH array. A second array platform is derived from SNP genotyping arrays. The addition of probes onto genotyping arrays that identify CNVs significantly enhances the sensitivity of detecting CNVs by genotyping array. The two array types show a functional convergence. Microarray platforms are able to detect large segments of cn-LOH and UPD, traditionally detected using microsatellite marker-based genotyping methods.[21] The one type of large-scale variant that is not detected using microarray techniques is balanced variants, and thus these variants can only be detected by conventional cytogenetic approaches. Due to the limited resolution of cytogenetic approaches, the balanced genomic variants are believed to be underdetected. It should be noted that many apparently balanced rearrangements ascertained by cytogenetic approaches are later found to harbor cryptic imbalances when high resolution microarray-based genomic profiling techniques are used.[22]

The advent of next generation sequencing (NGS) technologies has change the way genetic testing is performed and consequently brought a paradigm shift in genetic testing.[23] NGS-based whole exome sequencing (WES) or whole genome sequencing (WGS) generates DNA sequence data by massive parallel sequencing of clonally amplified or single DNA molecules.[24] Recent advances in NGS technologies are making an NGS-based diagnostic approach more cost-effective with a reasonable turnaround time.[25] Currently, gene panel testing for conditions with genetic heterogeneity or overlapping phenotype, and exome sequencing for conditions with complex presentation or uncertain clinical diagnosis, have become increasingly utilized as part of the genetic evaluation and even as first-tier test.[26] The premise for using WES instead of WGS for Mendalian disorders is that protein-coding sequences constitute about 1% of the human genome but harbor about 85% of the known variants that cause human diseases. However, given that large-scale variants, such as CNVs can be detected by a WGS, but not easily by WES, it is predicted that when the cost for WGS becomes acceptable for routine testing, it will become the method of choice for clinical diagnostics. Many challenges lie ahead, particularly in the area of data interpretation (informatics), which often involves database searching, segregation analysis, bioinformatic prediction, and functional demonstration.

NGS not only is effective for small-scale variants, but also it may eventually replace array-based technologies for interrogating CNV, as well as for many other types of large-scale variants. NGS data can provide a much more complete picture of cn-LOH in the whole genome.[27] NGS-based approaches are poised to solve effectively the technical difficulty of detecting balanced rearrangements; it will become the method of choice for genome-wide detections of both balanced and imbalanced genomic variants. One of the major hurdles in the application of NGS to genetic testing is informatics; how to understand and make sense of the vast amount of data generated. Finally, it should be noted that even though NGS will eventually replace many current genetic testing methods, conventional sequencing, genotyping, and CNV detecting technologies will continue to be useful for validating and confirming variants detected by NGS.

The nature of the variant dictates what method should be used for genetic testing. The clinician makes a clinical diagnosis and orders the gene test that is most likely to explain the patient's clinical condition. Conventional approaches to genetic testing in the clinical setting involve interrogating one gene or one variant at a time in one patient. The molecular diagnostic laboratory may use Sanger sequencing to test for small-scale variants, and whole gene or gene-panel based tests are currently the most commonly used approaches in genetic testing. As sequencing can only detect small-scale variants, it is often complemented by CNV testing using MLPA or quantitative PCR to detect potential exonic deletion or duplication. If a large-scale variant, such as a CNV is suspected, targeted methods for the detection may be performed using quantitative PCR or MLPA. Whole genome chromosomal microarray (CMA) analysis for CNVs and LOH is a useful scanning tool for genome-wide detection of possible genomic defects associated with many genetic conditions. In fact, the much enhanced clinical utility of CMA over conventional cytogenetic techniques justifies its use as the first tier test for patients with complex or

unknown genetic conditions, and microarray-based genomic profiling techniques have been recommended as the first tier genetic test in many clinical scenarios.

2 GENETIC TESTING FOR SKELETAL DISORDERS

2.1 Genetic Tests Available

As knowledge about genetic disorders of bone has expanded greatly over the past few years, the number of disorders for which genetic testing is commercially available has also increased (https://www.ncbi.nlm.nih.gov/gtr/). Although there has always been a lag between the research discovery of causative genes and the availability of commercial diagnostic testing,[28] genetic testing is available for a large number of skeletal diseases, including metabolic bone disorders and skeletal dysplasias.

Genetic testing for metabolic disorders of bone can be useful in the clinical setting for diagnostic purposes. There are a number of genetic causes of metabolic bone disease for which there is genetic testing available, including familial hypophosphatemic rickets, autosomal dominant hypophosphatemic rickets, two forms of autosomal recessive hypophosphatemic rickets , X-linked recessive hypophosphatemic rickets, and hereditary hypophosphatemic rickets with hypercalciuria. While X-linked recessive hypophosphatemic rickets is by far the most common, genetic testing for all of the forms is available (although genetic testing for hypercalciuria is currently only available clinically in Europe; see http://www.genetests.org) and can be used to differentiate between the forms when it is not clear from an inheritance pattern.[29] Genetic testing is available for most inherited vitamin D-related disorders, including vitamin D-dependent rickets type I (VDDR-IA) also known as 1-α-hydroxylase deficiency, and vitamin D-dependent rickets type II (VDDR-II) also called vitamin D-resistant rickets (http://www.genetests.org). Finally, genetic testing is available for hypophosphatasia, due to variants in ALPL, the gene encoding the alkaline phosphatase, tissue nonspecific isozyme. For many of these conditions, the diagnosis is made clinically and can be confirmed by genetic testing.

Genetic testing for skeletal dysplasias can be used to confirm a clinical diagnosis, as well as for cases where a clinical diagnosis is not immediately clear but several different disorders are under consideration. All patients with a suspected skeletal dysplasia should have a thorough physical exam documenting any dysmorphic features, as well as a full radiographic skeletal survey in cases where the diagnosis is not suggested by history and physical exam alone. There are hundreds of skeletal dysplasias and many genes that are affected. Here, we highlight some of the more common skeletal dysplasias for which there is genetic testing available.

2.2 When to Order Genetic Testing

Once a preliminary diagnosis of a genetic condition is made, the decision to order genetic testing is influenced by several factors, including the diagnostic and treatment necessity, the need for reproductive counseling, patient and family desire for testing, and cost and payment issues. Prior to ordering any genetic test, the provider needs to determine if the desired test is clinically available. In the USA, all genetic testing must be performed in a CLIA-certified clinical laboratory for both legal and reimbursement reasons.[30] The website http://www.genetests.org is an excellent source for determining which CLIA-certified clinical laboratories offer the desired testing. Any testing sent on a research basis must be done under the supervision of an institutional review board and often the results cannot be legally revealed to the patient until the testing is repeated and the results are verified in a CLIA-certified laboratory.[31] Fortunately, genetic testing tends to move rather quickly in most cases from the research setting to clinical testing.

The most common clinical situation where genetic testing is ordered is when the genetic testing is required to make a diagnosis or to confirm the diagnosis. Genetic testing is especially useful if a course of treatment is available and dependent on the diagnosis. In these cases, genetic testing will provide a clear benefit for the care of the affected patient. A clear example of this is osteogenesis imperfecta, in which bisphosphonates may be beneficial.

Often, if the diagnosis is unclear, a staged approach to testing, while perhaps prolonging the time to final diagnosis, can potentially conserve resources. This involves requesting one genetic test at a time and moving on to other genetic testing only if the initial tests are negative. Frequently, one blood sample can almost always provide enough DNA for a laboratory to carry out multiple sequencing analyses, thus preventing repetitive blood draws. This staged testing approach is also useful for disorders with genetic heterogeneity. Testing can begin with the most commonly affected gene and then proceed to subsequent genes only if the first test is negative. However, in cases where diagnosis will affect treatment and delaying therapy is not optimal, ordering multiple tests at once or gene panels may benefit the patient.

Reproductive counseling is another reason that genetic testing may be useful in a clinical setting. It is not uncommon for skeletal disorders to be diagnosed clinically based on family history, physical exam, and radiographic evidence. While the diagnosis may not be in question, knowledge of the specific variant causing the phenotype may be useful for future reproductive decisions. In the

pediatric setting, parents of an affected child may desire prenatal or preimplantation genetic testing with future pregnancies. In this situation, the patient's exact variant needs to be known to determine if the parents are carriers (or affected themselves if the variant is dominant) and to allow for prenatal testing with future pregnancies. In the case of recessive disorders, carrier testing is appropriate for adult family members but should not be performed on minor children until they reach maturity and can consent to their own testing.[32–35]

Patients or family members may request or refuse genetic testing for various reasons. In cases where genetic testing is needed for initial diagnosis or to confirm a clinical diagnosis, a full discussion of the risks and benefits should be undertaken with the patient (or parents if the patient is a minor). This discussion should include the reason(s) why genetic testing is needed, how are the results, whether a variant is found or not, will affect the patient's immediate after treatment, and how the results may affect the patient's care in the future. Discussion of these issues can help alleviate many misunderstandings and anxieties about genetic testing.[36] In the USA, the 2008 Genetic Information Nondiscrimination Act (GINA) bars discrimination by health insurance providers and employers due to genetic testing results, although GINA does not protect from genetic discrimination in other areas, such as long-term care or life insurance.[37] Finally, the discussion should include the limits of genetic testing, particularly when a negative test will not exclude a clinical diagnosis, which is often the case. Available genetic tests may only detect variants in a small number of clinically diagnosed patients depending on the disorder. Patients and families may consider a negative test result "the final answer" and need to understand that a negative genetic test does not always exclude a given clinical diagnosis.

The cost of genetic testing can affect the ability to order genetic tests in different settings. Genetic tests can be among the most expensive laboratory-based tests available, ranging from hundreds to tens of thousands of dollars. Third-party payers are becoming increasingly reluctant to pay for the costs of genetic testing, particularly in the absence of documented clinical necessity. In the pediatric population, single gene and panel testing for diagnostic purposes are often covered by private insurance companies, as long as the patient's pertinent history and physical findings are well documented. However, families with high-deductible plans should be warned that the cost of one genetic test may be greater than the entire deductible, requiring a single large payment. Carrier testing may not be covered by insurance companies, except when done for prenatal testing purposes. As genetic tests are ordered more frequently, institutions, such as hospitals may limit the number of tests that can be ordered or require that tests be ordered only from laboratories that bill the patients' insurance company directly. This can effectively put testing for some disorders out of the reach of patients and providers. These increasing costs place a responsibility on providers to ensure that genetic tests are only ordered under appropriate circumstances where there is a clear benefit for the patient.

The increasing availability of genetic testing for all types of skeletal disorders can be of great benefit to patients and providers. Genetic testing can be useful for diagnosis and subsequent treatment, as well as for reproductive counseling for patients and families. However, as with all medical testing, genetic testing has risks and benefits and may not be appropriate for every clinical situation.

3 GENETIC COUNSELING

There has been an "explosion" of new discoveries in the fields of genetics and genomics in the last decade that has resulted in the incorporation of new genetic tests, such as CMA analysis, gene panel testing, and whole exome/genome sequencing, into mainstream clinical practice. As much of the current research in genetics and genomics is focused on the study of complex trait versus single-gene disorders, the possibility that there will soon be numerous additional clinical tests available is not out of the question. Given the advances in genetic and genomic medicine, subspecialists, as well as primary care physicians have been increasingly being called upon to utilize genetic information, including risk assessment and testing in their practice[38,39] and help to determine which patients may be appropriate for genetic testing and counseling.

The diagnosis of a genetic disorder is likely to bring up numerous questions for patients and their families. Families must grapple with the many facets of the diagnosis, including the natural history, prognosis, and treatment options. "What caused this to happen?" and "Could this happen again?" are common questions asked in the setting of a new diagnosis as patients come to terms not only with their own diagnosis but also with the potential impact on other family members, both medically and psychosocially. Genetics professionals can help individuals to navigate these issues by providing the factual information required, as well as engaging patients and their families in a discussion of the potential psychological, social, and health impacts of the disorder. This affords the genetics provider the opportunity to better assess the patient's understanding of the information given and their ability to process that information to make autonomous decisions.

While primary care providers and subspecialists offer education concerning the clinical features, treatment, and prognosis of bone disease, genetics professionals are

often needed to ensure that patients and their families are receiving comprehensive information regarding the testing, therapeutic, and reproductive options available to them, as well as psychosocial support. As such, it becomes incumbent upon primary care clinicians, genetics professionals, and other clinical specialists to partner to provide optimal genetics care, including counseling, to their patients.

3.1 History

The field of genetic counseling is fairly young compared to other healthcare professions, albeit one that continues to grow rapidly. The term "genetic counseling" was coined in 1947 by Sheldon Reed, who subsequently authored the book *Counseling in Medical Genetics*.[40,41] At the time of his publication in 1955, there were approximately 12 genetics clinics in North America providing services to families.[42] Reed proposed providing genetic information to families while remaining attune to their psychological needs.[40,43] However, the history of the field of genetics shows the limited scope of information that could be provided to patients during the 1940s and 1950s. In fact, the structure of DNA remained unknown until 1953, while the diploid number of chromosomes was not discovered until the publication by Tjio and Levan in 1956.[44] In addition, it would be another decade before the successful use of amniocentesis for genetic diagnosis was demonstrated. Therefore, in the early years of genetic counseling, families could only be provided with medical information and predictions of recurrence risk. Over the ensuing decades, the identification of cytogenetic abnormalities and single gene disorder variants, as well as the development of prenatal diagnostic procedures greatly expanded the need for skilled professionals to provide genetic counseling to patients and families.

Genetic counseling is carried out by genetics professionals, generally clinical geneticists and genetic counselors. Genetic nurses credentialed through nurse genetics programs (http://www.isong.org/ISONG_genetic_nurse.php) may also provide genetic counseling. In the USA, clinical geneticists are physicians who have completed a minimum of 1 year, full-time training that includes at least 12 months of direct patient care experience in an American Council of Graduate Medical Education accredited residency, and then at least 24 months of training in an American Council of Graduate Medical Education-accredited Clinical Genetics and Genomics training program. Alternatively, physicians can complete a 4–5 year training residency programs that combines Clinical Genetics and Genomics with Internal Medicine, Maternal Fetal Medicine, Reproductive Endocrinology and Infertility, or Pediatrics. Clinical geneticists are trained to provide "comprehensive genetic diagnostic, management, therapeutic, and counseling services" (http://www.abmgg.org/) and are certified by the American Board of Medical Genetics.

Genetic counselors are trained in Master's-level genetic counseling training programs[45] accredited by the Accreditation Council for Genetic Counseling (http://www.gceducation.org) and are currently certified by the American Board of Genetic Counseling (http://www.abgc.net). At present, 19 states within the USA also require licensure of genetic counselors, a number that is growing steadily. Graduates of these genetic counseling programs are trained to provide services to patients and their families, and receive instruction in human, medical, and clinical genetics, as well as counseling theory and techniques. The training of all genetics professionals also includes exploration of the relevant social, ethical, and legal issues inherent in the field.

As noted earlier, practitioners of genetic counseling or "genetics professionals" include genetic counselors, clinical geneticists, genetic nurses, and other clinicians with specialized training in the field. While genetics providers have traditionally worked in academic medical institutions, genetics professionals also provide services within the primary care, diagnostic laboratory, and public health sectors, as well as in specialized areas, including assisted reproductive technologies, cardiology, and oncology. As such, genetics providers serve patients at all stages from preconception through pregnancy to pediatrics through adulthood.[46]

3.2 The Genetic Counseling Process

Genetic counseling, as defined by the National Society of Genetic Counselors,[47] "… is the process of helping people understand and adapt to the medical, psychological and familial implications of genetic contributions to disease. This process integrates the following:

- Interpretation of family and medical histories to assess the chance of disease occurrence or recurrence.
- Education about inheritance, testing, management, prevention, resources, and research.
- Counseling to promote informed choices and adaptation to the risk or condition."[47]

The technique of "nondirectiveness" has been intertwined with genetic counseling since its inception. However, this approach has been questioned, both in terms of its definition, as well as its role in the genetic counseling session.[48,49] Kessler states that nondirectiveness "describes procedures aimed at promoting the autonomy and self-directedness of the client." With this in mind, professionals engaged in genetic counseling strive to provide information to patients in a noncoercive and empathetic manner to facilitate autonomous decision making. Whenever possible, information is given in a

way that is unbiased. However, there may be situations when this may not be appropriate, such as discussing a particular diagnosis that has a well-established treatment strategy (e.g., calcitriol for vitamin D-dependent rickets, type I) or a surveillance program (e.g., tumor screening through imaging and biochemical testing in the setting of MEN1).[50] Genetics providers have a responsibility to provide professional guidance and should be comfortable advising their patients to consider the treatment or surveillance program once the benefits, risks, and limitations have been fully described. This does not mean that a counseling session will be devoid of advice given in a noncoercive manner, if that is what is required based upon the patient's needs and circumstances.[48,51] Ultimately, the counseling provided should be skills-based, and remain flexible to be effective in any given session.[48,49,51]

Regardless of the setting, the key components of a genetic counseling session remain largely the same—information gathering, establishing or verifying diagnosis, risk assessment, information giving, psychological assessment and counseling, help with decision making, and ongoing client support.[51,52] The genetic counseling session is specific to each patient in terms of providing information in a culturally sensitive manner by being attuned to a patient's educational and socioeconomic background. Techniques to achieve this include the use of layman's terms, avoidance of words that are emotionally charged, such as "abnormal" or "variant," and the preferred use of person-centered language (e.g., child with achondroplasia vs. achondroplastic child) to emphasize the individual person not the disorder. Visual aids are often useful to better illustrate the information provided. It is of the utmost importance that the counseling be consistent with the patient's perception and needs, to ensure that the patient's ability to successfully utilize the information for informed decision making is not impaired.

3.3 The Genetic Counseling Session

Genetic counseling is indicated once the diagnosis or presumed diagnosis of a genetic condition is made, either by a clinical geneticist, primary care physician, or subspecialist. A patient may be referred to the genetics professional with a diagnosis that needs to be confirmed, or the patient may be referred to a clinical geneticist to make a genetic diagnosis. Regardless of the setting, the components of the genetic counseling session are similar. The session begins with the genetics professional contracting with the patient, including solicitation of the patient's understanding of the reason for referral and expectations/desired outcomes of the session. This allows the genetics provider to dispel any initial misconceptions regarding the purpose and potential outcomes of the genetic counseling session including genetic testing.

3.3.1 Family History

Eliciting the family and medical history information and constructing the pedigree using standard nomenclature is central to the genetic counseling session.[46,53] While the pedigree is necessary for determining the pattern of inheritance and assessing recurrence risks, it can also be helpful in determining family dynamics through exploration of the patient's psychosocial history. Indeed, the patient's family history should engage in a discussion of the personal, family, and community values to help to appreciate the patient's support network and coping skills while identifying areas of concern. The "pedigree process" also allows one to correct any misapprehensions the patient may have about the degree of heritability in a family for any given diagnosis. Families may have long-held beliefs regarding the causes of certain disorders within the family. For example, family members may falsely attribute birth trauma as the cause of a disorder, or believe that a certain disorder affects only males since all of the females in their family are unaffected. Therefore, it is important that the genetics provider initially determines how the family has come to such beliefs to address any misconceptions in a sensitive yet effective manner.

The duty to warn relatives with respect to their genetic risk is an ethical issue that often needs to be addressed during this portion of the genetic counseling session. This duty may bring up a multitude of concerns, including patient confidentiality and the autonomy of relatives in being able to choose not to know of their risk status.[54] Given the current status of privacy and confidentiality legislation, genetics professionals cannot directly contact other family members deemed at risk. However, as the discussion of the family history includes the possible identification of at risk family members not present in the session, the potential impact the disorder may have on the medical management of those family members cannot be ignored. While the focus of the counseling session should remain on the patient and the family members present, genetic counseling needs to include guidance as to why and how to discuss the diagnosis and potential impact with other family members, and to guide them to appropriate resources.

3.3.2 Genetic Testing

The genetic counseling session often includes a discussion of the availability of genetic testing or lack thereof. One of the core tenets of genetic counseling is the belief that the decision of whether or not to proceed with genetic testing is voluntary. As such, patients must be counseled as to the real and potential risks, benefits, and alternatives to testing, and also be given the assistance needed to make an informed decision regarding their testing options. It is worth noting that patients may actually put more weight on their perceived risk and

emotions as opposed to the factual risks.[55] Given this, and the fact that genetic test results may impact not only the patient but other family members, it is preferred that a patient receive genetic counseling prior to genetic testing. Pretest genetic counseling also allows for a discussion of the potential for unclear or unexpected findings, such as variants of unknown significance, incidental/secondary findings, and nonpaternity.

Genetic testing in the setting of childhood and adolescence is an issue that continues to be debated in the genetics field. Multiple professional societies have taken the stance that testing of minors should only be done when the disorder in question has a childhood onset and effective treatment or surveillance in childhood is available.[33,35,54,56,57] Although recently the American College of Medical Genetics and Genomics released a policy statement stating that incidental findings including those of adult onset identified in pediatric patients be disclosed to families in the setting of clinical exome and genome sequencing.[58,59] This was not without controversy.[60,61] However, there remains a lack of consensus as to the definitive steps to take in the setting of a disorder without preventative or therapeutic options.[62,63] There are some instances in which the appropriateness of genetic testing in childhood and adolescence seems quite clear. For example, genetic testing for MEN Type II via RET variant analysis for minors with a substantial risk (50%) is an accepted practice as thyroidectomy to prevent medullary thyroid carcinoma should be performed during childhood.[64] On the other hand, although diagnostic testing for an autosomal recessive skeletal dysplasia in a child is considered appropriate for surveillance purposes and for assessing recurrence risks in the parent, carrier testing of siblings may not be appropriate given the lack of empiric evidence regarding the effect that presymptomatic or carrier testing may have on children.[65]

When offering genetic testing to minors, it is important for parents and their children to be fully informed of the potential benefits, risks, and limitations of genetic testing, including a discussion of the child's autonomy and privacy issues. The potential benefits, such as elimination or reduction of uncertainty, or the initiation or elimination of surveillance techniques, must be weighed against the potential harms, such as increased anxiety or effects on familial relationships. If the patient is not of the age and/or level of maturity to assent to carrier or presymptomatic testing, parents should be engaged in a discussion of when and how they will inform their child of the test results (whether they are negative, positive, or inconclusive) and their reasons for pursuing testing at that time in their child's life.

Another issue that has been highlighted with regard to genetic testing is the "duty to recontact." It is not unimaginable for a patient to receive genetic counseling at a time when genetic testing for their specific disorder is not yet possible, but several months or years later the causative variant is identified and clinical testing becomes available. Patients may also need to be recontacted in the setting of previously identified variant of unknown significance being reclassified as a causal or "normal" variant. Legal and ethical arguments have been raised for and against the duty to recontact when new information is discovered regarding a genetic test or diagnosis.[66–68] Some have advocated that it is the treating clinician's responsibility to initiate recontact whereas others have suggested that the responsibility lies, at least partially, with the patient. The issue of practicality also comes into play. Does a genetics clinic have the appropriate infrastructure and resources required to recontact all relevant patients with new testing options? How will the primary care provider keep current information on the development of new genetic tests? Currently, as outlined by the American College of Medical Genetics and Genomics policy statement, the genetics provider should initiate the recontact in cases where there is an ongoing relationship with the patient, whereas in cases lacking this ongoing relationship, the primary care provider should shoulder the responsibility.[67] The policy further recognizes the importance of encouraging patients to contact their primary care or genetics provider if, or when, changes pertinent to their diagnosis arise.

The importance of a client's confidentiality and privacy, including testing results, has been acknowledged within the field of medicine for some time. Genetic testing poses unique challenges as the disclosure of genetic information, whether voluntary or involuntary, has the potential to lead to social and economical consequences. The passage of GINA was landmark legislation for the field of genetics, as it affords protection from workplace and health insurance discrimination. However, as noted previously, it does not speak to the possibility of genetic discrimination with regard to life, disability, or long-term care insurance. As such, the potential for insurance discrimination based on one's genetic background is a topic, which is still discussed during the genetic counseling session, particularly when patients are contemplating presymptomatic genetic testing.

3.3.3 *Communicating the Facts*

Patient education and disclosure of information go hand in hand. Genetics providers operate under the belief of complete disclosure; families should be given all relevant information as it pertains to the diagnosis.[51] Complete disclosure, however, is subjective; as what is deemed relevant information by a given practitioner or a family may well differ from another. In addition, it may not be appropriate or feasible to disclose every piece of information regarding a disorder, genetic test, or treatment as the provision of too much information during a single session may lead to confusion and negatively

impact decision making. For example, when providing prenatal genetic counseling to a couple who already have a child with a skeletal dysplasia, a detailed explanation of prenatal testing options may be more appropriate than a lengthy discussion of the prognosis of the disorder. Alternatively, in the pediatric setting, an extensive description of prenatal diagnostic testing may not be appropriate when the family is primarily interested in confirming the diagnosis and obtaining prognostic information for their child. Indeed, genetic counseling aims to provide patients with the information they will need to make the most informed and autonomous decision possible at that time.

Dissemination of information also includes a discussion of "risks," whether it is recurrence, genetic testing, or prenatal diagnostic procedures risk. In genetic counseling practice, as the term "risk" may be seen as a negative term, the terms "chance" or "likelihood" are often preferred.[51,69] However, people have difficulty quantifying their own risks accurately, which has been well-documented, including the trend toward overestimation.[70] Whether the use of the word "risk," when one actually means "probability," has lent itself to oversimplifying patients' risk perception and their subsequent decision making has also been questioned. The ability of a patient to recall the quantitative risk figure they were given may not provide insight into how they perceive that risk, nor the steps they may take to mitigate the risk. Thus, genetic counseling should focus on understanding how the risk is internalized by the patient rather than the regurgitation of the risk figure.

People process risk figures differently. Therefore, risks should be provided using a variety of techniques, including fractions, percentages, and scales. A patient with a 1% risk for recurrence in their offspring may be counseled that, "You have a 1% chance for this to occur in any future pregnancy which means you also have a 99% chance that it will not occur." Illustrating both sides of the risk (the chance something will happen and the chance it will not) may help patients to better understand the degree of risk. In addition, the use of more than one method of risk framing (e.g., using percentages and fractions) to describe the same risk figure may be beneficial. For example, a genetics provider may counsel a patient that "A 1% chance means that 1 out of 100 people will develop the disorder while 99 out of 100 people will not."

Patients take many factors into account when assessing their risks, including their personal experiences with the condition, environmental factors, occupation, and diet, to name a few.[70] One's physical resemblance to an affected family member may also influence an individual's personal risk assessment.[70] Additionally, a patient's perception of risk can also change over time as one's life experiences change.[71] As people's perception of risk influences their future actions, genetics providers need to ensure that this perception is not skewed in a particular manner and also provide support as patients internalize and manage their risk. Following the risk dissemination, patients should be asked questions, such as "Is this a number you expected?" and "Does this seem like a high, low or average chance?" One cannot make assumptions, as a 2% risk may be deemed a high risk by one individual while another may perceive it as negligible. By asking such questions, the genetics professional can ascertain whether the patient has understood the risk and how it is personally perceived. This helps to illuminate the patient's understanding of the information provided and allow for adjustment of counseling as needed.

3.3.4 Counseling and Support

During the counseling session, a focused assessment of the patient's psychosocial needs must be performed. In fact, it has been suggested that in some cases the emotional support provided during the counseling session may be more beneficial to clients than the factual information presented.[72] Patients may express a variety of emotions during the genetic counseling session, including anger, grief, denial, guilt, shame, helplessness, and fear of social discrimination.[51–53] Genetics providers need to determine how the patient will cope with the information to foster independent decision making. Again, it is important for genetics providers to remember that how a patient views risks, makes decisions, adapts to situations, and communicates within a family depends largely on life experiences and cultural background.

As previously stated, patients must often make decisions based on the factual information provided to them during the genetic counseling session. Such decisions may involve choosing a course of treatment, pursuing or declining genetic testing, making reproductive choices, or informing others of the diagnosis and its implications. The genetics provider must strive to empower patients in their decision making. Potential obstacles to autonomy should be identified and assistance provided to reduce or eliminate those barriers. To help patients determine the best course of action for their family at that time, patients should be encouraged to explore various scenarios envisioning how different decisions may affect their family socially, economically, and emotionally. The patient's thoughts and feelings about the diagnosis should be addressed, not only to assess comprehension, but also to determine if or how this may affect their ability to inform other family members, friends, coworkers, etc. of the situation. Patients should also be engaged in a discussion of the potential responses, both verbal and nonverbal, they may encounter when informing others of the diagnosis and their decisions surrounding it. The patient's potential reactions to such responses should also be explored along with their coping strategies to effectively handle such responses.

Providing assistance and support to the patient is vital to information processing and independent decision making. As such, genetics providers need to identify the additional resources that patients and their families may require as they process and incorporate the information given. A patient may benefit from participation in a formal support group or a one-on-one interaction with another patient with the same diagnosis. In the prenatal setting, it may be helpful for a patient to speak to other individuals who had been given a similar prenatal diagnosis and grappled with their reproductive options. Genetic counseling must also include the facilitation of referrals to other healthcare providers for further information regarding treatment, prognosis, and other medical information as needed. Additionally, genetic providers often offer information regarding disorder specific foundations and/or genetic organizations (e.g., Osteogenesis Imperfecta Foundation, Paget Foundation, Genetic Alliance), which can provide supplemental information and support to patients and their families. Furthermore, genetics professionals must recognize when a patient may require additional psychological support outside of the genetic counseling session and refer patients when appropriate.

Significant advances continue to be made in genetics and genomics including the increased availability of clinical testing. As such, the field of genetic counseling has had to keep pace with this progression, oftentimes resulting in more challenging genetic counseling sessions. Genetics professionals remain at the forefront of this genetics and genomics revolution, ensuring that patients are adequately counseled as to the many facets of their particular genetic diagnosis while also providing the support needed for successful information processing and autonomous decision making.

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SECTION 2

GENERAL BACKGROUND TO BONE BIOLOGY

CHAPTER

11

Biology of Bone and Cartilage

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1 INTRODUCTION

Bones are formed during embryonic development through endochondral and intramembranous ossification. Long bones are formed by endochondral ossification from limb buds that extend from the embryonic torso and form cartilaginous molds (known as anlagen), which are shaped roughly in the form the bones will have when they are more fully formed. The chondroblasts that give rise to these anlagen are derived from mesenchymal stem cells (MSCs), which also give rise to osteoblasts (OBs), fibroblasts, adipocytes, striated muscle, and endothelial cells, which comprise most of the tissues in limbs.[1,2] Cells in the central parts of the anlagen differentiate from small prehypertrophic into large hypertrophic chondrocytes, and the matrix around these cells calcifies by means of a process that remains poorly understood.[1] This calcified cartilage is invaded by blood vessels, associated with matrix dissolution by metalloproteinases and chondrocyte apoptosis. As the calcified cartilage is removed, it is replaced by new bone laid down as plate-shaped structures called trabeculae by teams of OBs derived from precursors, some of which appear to circulate in the blood.[3] Some of the OBs on the surface of the new trabeculae become embedded in the unmineralized matrix (osteoid) and remain there as osteocytes as the matrix calcifies and until the bone is subsequently remodeled. Osteocytes have numerous functions within and outside of bone, some anticipated and others unexpected.[4]

Radial growth of long bones and growth of flat bones, such as some of the bones of the skull, are accomplished by intramembranous ossification in which bones are formed directly by OBs without being preformed in cartilage. As bone growth and the formation of a bone marrow (BM) cavity within each long bone occurs very rapidly during embryonic development, new bone has to be removed quickly by osteoclasts (OCs) to prevent the cavity being filled with unresorbed bone. Failure of OC formation or function during skeletogenesis results in buildup of bone in BM cavities, called osteopetrosis,[5,6] which can limit movement of hematopoietic precursors formed in the liver to the BM and the transition from extra- to intramedullary hematopoiesis. Removal of calcified hypertrophic cartilage in anlagen may involve OCs, but these cells are not required, since it occurs in the absence of OCs in osteopetrotic humans and other mammals[2,7] and the mechanisms involved are poorly understood. The matrix may be dissolved by metalloproteinases secreted possibly by the chondrocytes themselves or by endothelial cells in nearby vessels or by chondroclasts, poorly characterized cells that may be in the mononuclear/phagocyte lineage.[2] OCs are often mistakenly assumed to be chondroclasts because they resorb at the osteochondral junction in growth plates. However, chondroclasts are not multinucleated and do not express tartrate-resistant acid phosphatase (TRAP) in sections of bones from osteopetrotic mice, such as NF-κB double knockout (dKO) and $RANK^{-/-}$ mice that do not form OCs or $TRAP^{+}$ mononuclear cells in bone. These KO mice still have vascular invasion of anlagen, removal of cartilage, and formation of bone, but this bone does not get resorbed.[2,7]

There is continuous modeling and remodeling of the shape and thickness of bones during childhood and adolescence in response to patterning and mechanical stimuli. After skeletal maturity is attained, bone integrity is maintained by continuous, highly regulated, bone remodeling, during which OCs excavate trenches on bone surfaces and tunnels through cortical bone that are subsequently filled by new bone laid down by OBs. New bone consists of layers (lamellae) of extracellular matrix of primarily type I collagen upon which a crystalline inorganic component (predominantly calcium hydroxyapatite) is laid down during mineralization. Sites of bone remodeling have been defined as basic multicellular units or bone remodeling units. Many metabolic, inflammatory, and neoplastic conditions affect bones

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and joints. They impact the remodeling cycle and can positively or negatively affect turnover rates and thus bone volume and shape. Bone modeling and remodeling are regulated locally by interactions between cells, by factors released from cells that influence functions of cells around them, and systemically by hormones and growth factors released by other organs, as well as by the central and peripheral nervous and intestinal systems. Given this complexity, it is not surprising that there are numerous congenital disorders of skeletal development.

In this chapter, we review current understanding of how bone cells are formed and perform their functions, interact with one another to form and maintain bones, and how deficiency, mutation, aberrant, or overexpression of the genes that encode the proteins involved in these functions can lead to common and rare bone diseases.

2 OSTEOCLASTS

OCs are multinucleated cells formed by cytoplasmic, but not nuclear, fusion of committed precursors derived from myeloid lineage hematopoietic cells that also give rise to macrophages.[2] Differentiation of the progenitor cells requires their expression of cytokine receptors and numerous transcription factors and signaling molecules that regulate osteoclastogenesis. Macrophage-colony stimulating factor (M-CSF) and RANKL are the two most important cytokines required for OC formation under basal and pathologic conditions,[2,7] but costimulatory signaling through other receptors also mediates osteoclastogenesis, particularly in response to inflammation[8] OCs have long been considered to be merely bone-degrading cells whose formation and functions were under the control of osteoblastic, stromal, and immune cells in the marrow. However, OCs and their precursors (OCPs) interact with and positively and negatively regulate the formation and functions of these and other cell types, and they can behave as immune cells.[9] These findings and reports of OCs having regulatory roles in autoimmune and inflammatory diseases affecting the skeleton spawned the field of osteoimmunology.[8] Murine and human genetic studies have identified several genetically inherited diseases that result from mutations in genes that regulate the formation and activation of OCs. This chapter will focus on current knowledge of the genes and proteins that regulate these processes and how defects in them affect the skeleton.

2.1 Regulation of Osteoclast Formation: The RANKL–RANK–OPG Pathway

The RANKL/RANK/NF-κB signaling system and osteoprotegerin (OPG), which positively and negatively regulate the mid-to-later stages of osteoclastogenesis were discovered >20 years ago.[2,10] These seminal studies revealed that RANKL$^{-/-}$ and transgenic mice overexpressing OPG had marked osteopetrosis because they lacked OCs, while OPG$^{-/-}$ mice had severe osteoporosis due the unopposed OC-inducing effects of RANKL. Mice deficient in NF-κB1 and 2, two major components of the NF-κB signaling pathways that regulate immune responses, were reported earlier to have marked osteopetrosis due to a lack of OCs.[2] Later, it became clear that NF-κB signals downstream from RANK, and is required for RANKL-induced OC formation.[2,10]

RANKL is a member of the TNF superfamily of proteins and is typically expressed on the surface of osteoblastic cells in response to most of the factors known to stimulate bone resorption. However, like M-CSF, it is also secreted by osteoblastic cells and by numerous other cell types, including activated T cells,[2,10] B cells, and synovial cells in joints of patients with rheumatoid arthritis (RA) where, along with other cytokines, it contributes to joint destruction,[2,10] and where synoviocytes appear to be the major source of RANKL.[11] Chondrocytes in growth plates express RANKL, RANK, and OPG.[2] 1,25-dihydroxyvitamin D, bone morphogenetic protein 2 (BMP2) and Wnt/β-catenin signaling regulate chondrocyte expression of RANKL to attract OCPs to growth plates for removal of newly formed bone.[2] RANKL$^{-/-}$ and RANK$^{-/-}$ mice, like NF-κB1/2 dKO mice, also have impaired B-cell development, and failure of lymph node formation[2] and mammary lobular hyperplasia during pregnancy leaving them unable to feed their offspring.[12] RANKL centrally controls fever and body temperature in females[12] and promotes mammary cell proliferation,[12] carcinogenesis, and breast cancer metastasis to bone.[12] It also has antiinflammatory and neuroprotective effects in a mouse model of ischemic stroke in which it limits infarct volume.[12] Mutations in RANKL occur rarely in humans associated with osteopetrosis, but affected individuals do not appear to have obvious immunological defects.[13]

RANK, a TNF receptor superfamily member, is expressed by OCPs in response to PU.1, microphthalmia-induced transcription factor (MITF), M-CSF, TNF[2], Il-34,[14] and Wnt5a,[15] thus priming them for terminal differentiation when they encounter RANKL. RANK is also expressed by dendritic cells, normal breast epithelial cells,[12] and in some cancers, including breast and prostate.[12] Inactivating mutations of *rank* are rare in humans and are associated with OC-poor osteopetrosis.[13,16] Activating *rank* mutations are more common[13] and account for the increased OC formation, activity, and osteolysis seen in some patients with early-onset (juvenile) Paget's disease, expansile skeletal hyperphosphatasia, and familial expansile osteolysis.[5,6]

OPG binds to RANKL and prevents its interaction with RANK and downstream signaling through TNF receptor-associated factor 6 (TRAF6) (Fig. 11.1). It is

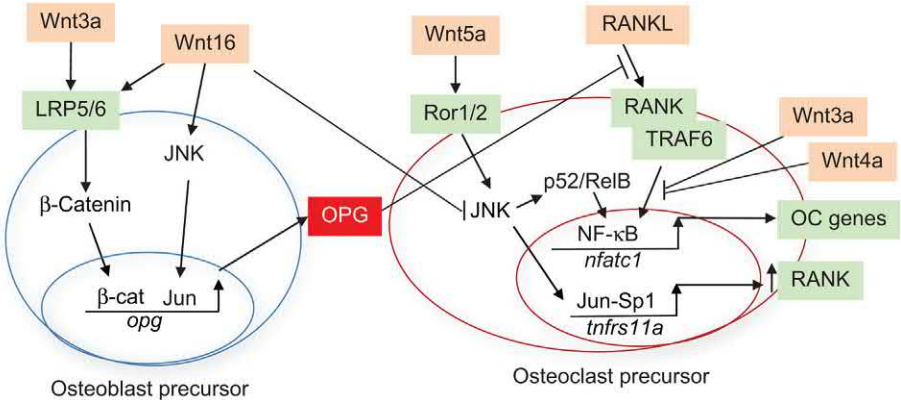

FIGURE 11.1 Wnt regulation of osteoclastogenesis through direct and indirect mechanisms. Wnt3a and Wnt16 inhibit osteoclast (*OC*) formation by upregulating *opg* expression in osteoblastic cells via β-catenin in the Wnt canonical pathway. Wnt16 also upregulates *opg* expression through Jun kinase/Jun signaling in the noncanonical pathway; in addition, Wnt16 expressed by osteoblastic cells also limits OC formation and activation directly by inhibiting RANKL-induced JNK/NF-κB-mediated *nfatc1* expression. In contrast, Wnt5a, also expressed by osteoblastic cells, increases *tnfrs11a* (*rank*) expression via Ror2/JNK signaling in the noncanonical pathway, leading to enhanced OC formation. Wnt4 inhibits RANK/TRAF6-induced expression of NF-κB-mediated *nfatc1*, which Wnt3a also inhibits.

secreted by OBs in response to most of the factors that also upregulate RANKL expression and in this way it limits OC formation, activity and survival.[12] Cells in numerous other organs express OPG, including the liver, heart, spleen, and kidney, where it has other regulatory functions. Some patients with juvenile Paget's disease, an autosomal recessive disorder,[17] have homozygous partial deletions of *opg* resulting in osteopenia and fractures. Some children with idiopathic hyperphosphatasia, an autosomal recessive disease associated with increased bone turnover and deformities of long bones, acetabular protrusion, and kyphosis, have an inactivating deletion in exon 3 of *opg*.

Major pathways that regulate OB formation and differentiation of MSCs to OBs also affect RANKL/RANK and/or OPG expression and OC formation. For example, Wnt3a- and Wnt16-induced canonical signaling through β-catenin upregulates OPG expression in OBs[18] (Fig. 11.1). Wnt16, Wnt3a,[19] and Wnt4a[20] expressed by osteoblastic cells also limit OC formation noncanonically by inhibiting RANKL-mediated noncanonical NF-κB-induced NFATc1 expression (Fig. 11.1). Wnt5a, also expressed by osteoblastic cells, positively regulates OC formation noncanonically through receptor tyrosine kinase (TK)-like orphan receptor (Ror) proteins expressed on OCPs that leads c-Jun N-terminal kinase (JNK)-mediated increased expression of RANK[15] (Fig. 11.1). Importantly, a soluble form of Ror2 acted as a decoy receptor of Wnt5a and abrogated bone destruction in mouse models of arthritis.[15] Activation of β-catenin signaling in early OCPs promotes their differentiation into OCs, but activation of this pathway in more differentiated precursors inhibits OC formation[21] Thus, Wnts can have positive and negative regulatory roles in osteoclastogenesis. In addition, Jagged1/Notch1 signaling, which also regulates MSC numbers and OB differentiation, negatively regulates OC formation indirectly by affecting the OPG/RANKL ratio in stromal cells.[2,22,23] However, the effects of Notch ligands and receptor signaling on osteoclastogenesis are complex and may be time- and context-dependent.[24]

OPG$^{-/-}$ mice also have aortic and renal artery medial calcification,[12] which complicates atherosclerotic plaques on the intimal surfaces of arteries, particularly in patients with diabetes mellitus and/or chronic renal failure. OPG has been implicated in calcification because OPG/apoE dKO mice have more accelerated calcific atherosclerosis than apoE$^{-/-}$ mice.[2,12] Aberrant OPG and RANKL expression have been implicated in cardiovascular disease,[25] diabetes, hypertension, and numerous other diseases, suggesting that OPG or RANKL inhibitors could be used to limit calcification of arteries in these common diseases.

2.2 Transcription Factor Regulation of Osteoclastogenesis

Transcription factors with essential functions in hematopoiesis, including PU.1, Pax5, and NF-κB, also regulate multiple stages of OCP differentiation. PU.1$^{-/-}$ and Pax5$^{-/-}$ mice, like NF-κB1/2 dKO mice, do not form OCs and develop marked osteopetrosis.[2,16] PU.1 along with MITF regulates expression of c-fms, the M-CSF receptor in OCPs, and M-CSF induces RANK expression in OCPs by upregulating c-Fos expression. However, M-CSF alone is unable to drive complete progenitor cell differentiation into OCs, which requires exposure to RANKL and activation of c-Fos and NFATc1 through NF-κB[2] (Fig. 11.2). NF-κB1/2 dKO mice have increased numbers of CD11b + /RANK + OCPs[2], similar to RANKL$^{-/-}$ mice; if treatment with Denosumab,

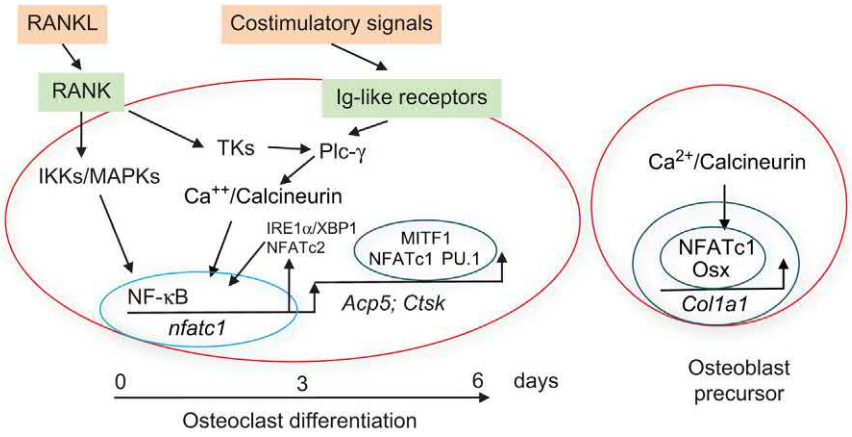

FIGURE 11.2 Activation of NFATc1 in osteoclastic and osteoblastic cells. RANKL/RANK signaling activates NF-κB to induce very early expression of NFATc1, which transiently autoamplifies its own expression through NFATc2 and IRE1α/XBP1 and induces OCP differentiation. NFATc1 is also activated by costimulatory signaling and by RANK through Brouton and Tec tyrosine kinases (*TKs*), which activate Plcγ. This leads to intracellular Ca^{2+} fluxes, which activate calcineurin-mediated NFATc1 dephosphorylation and NFATc1 nuclear translocation. In the later phases of OC formation, NFATc1 binds to the promoters of TRAP (*Acp5*) and cathepsin K (*Ctsk*) in a complex with MITF and PU.1 to promote their expression and bone resorption. NFATc1 also induces expression of genes involved in OCP fusion, including DC-STAMP, OC-STAMP, and Atp6v0d2, as well as intergrin β3 and MMP9. NFATc1 is also involved in osteoblast (*OB*) differentiation by binding to and synergizing with osterix on the *Col1a1* promoter to enhance its expression.

a RANKL monoclonal antibody, increases CD11b+/RANK + OCP numbers in humans, rapid differentiation of these cells into OCs with accelerated resorption could potentially explain the increased resorption marker and fracture rates that have been reported following cessation of therapy in some osteoporotic patients.[26] NFATc1 is activated by RANK and costimulatory signaling following its dephosphorylation through a calcium-dependent mechanism involving PLCγ and calcineurin[12] (Fig. 11.2) and regulator of G-protein signaling 10 (RGS10), which interacts with Ca^{2+}/calmodulin and phosphatidylinositol (3,4,5)-triphosphate (PIP_3) to mediate PLCγ activation and $[Ca^{2+}]_i$ oscillations in OCPs.[27] Ca^{2+}/PLCγ signaling can also be activated by adenosine, which is generated from the catabolism of adenine nucleotides in response to oxidative stress and hypoxia. Blockade or deletion of adenosine receptor A_1R reduced bone resorption in mice, resulted in osteopetrosis, while $A_{2A}R^{-/-}$ mice have increased bone resorption and reduced bone mass. An $A_{2A}R$ agonist reduced wear debris-induced bone resorption and inflammation in mice, suggesting that stimulation of $A_{2A}R$ signaling specifically might diminish orthopedic prosthesis loosening.[28]

Ca^{2+} oscillations occur through Ca^{2+} release from mitochondria and are regulated by transmembrane protein 64 (Tmem64), which interacts with sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase 2 (SERCA2).[29] This leads to increased Ca^{2+}/calmodulin-dependent protein kinase (CaMK) IV activity and mitochondrial release of reactive oxygen species, both of which are required for osteoclastogenesis. $Tmem64^{-/-}$ mice have increased bone mass due to decreased OC formation, associated with suppression of CREB activation and c-Fos and NFATc1 induction. However, they also have increased bone formation, associated with increased osteoblastic expression of Runx2, osterix and β-catenin, and reduced adipogenesis.[29] Thus, Tmem64 appears to positively regulate bone resorption and limit bone formation. Limiting its expression or enhancing its degradation could increase bone mass.

Additional factors that activate NFATc1 include myostatin, which is a TGFβ superfamily member expressed by myocytes that negatively regulates muscle growth and regeneration after injury. Myostatin is also secreted by synovial fibroblasts in inflamed RA joints and by osteoblastic and osteoclastic cells. It increases osteoclastogenesis and bone resorption by paracrine and autocrine actions through SMAD2-enhanced NFATc1 nuclear translocation and activation (Fig. 11.3) resulting in increased expression of integrin αv, integrin β3, DC-STAMP, and calcitonin receptor, and in OC numbers and size.[30] Myostatin deficiency or inhibition reduced the severity of arthritis in TNF-transgenic mice, mainly through decreased bone erosion.[30] In response to RANKL, the transcription factor, c-myc, directly binds to the NFATc1 promoter[31] in association with bromodomain and extraterminal (BET) proteins to promote NFATc1 expression (Fig. 11.3). BET proteins bind to acetylated histones (H-Ac) on chromatin via their bromodomains and epigenetically regulate gene transcription by recruiting additional chromatin regulators. A small molecule BET inhibitor prevented c-myc binding to NFATc1 and reduced OC formation and OVX-induced and inflammatory bone loss.[31] NFATc1 is also activated transiently in association with Ca^{2+} oscillations and ER stress in OCPs by the inositol-requiring protein-1α/X-box–binding protein1 (IRE1α/XBP1)-mediated branch of

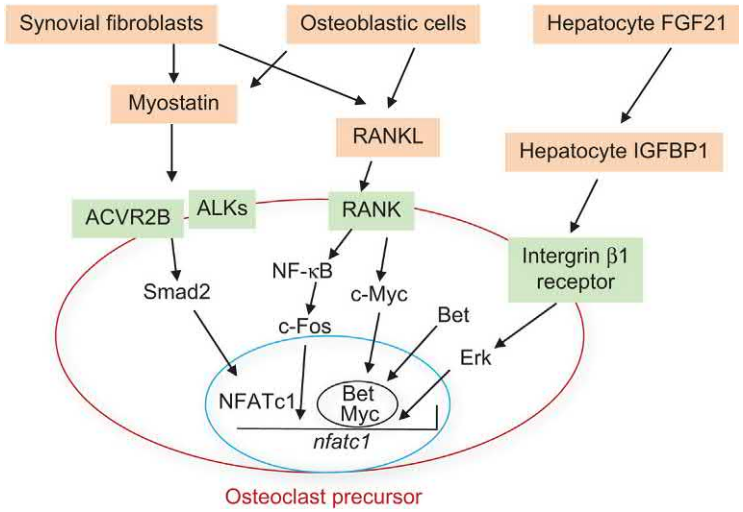

FIGURE 11.3 Regulation of NFATc1 expression in osteoclastic cells by cells outside of bone. OB precursors, osteocytes and synovial fibroblasts express RANKL, which induces NFATc1 nuclear translocation and increases *nfatc1* expression through RANK/NF-κB/c-Fos signaling. RANK signaling also induces nuclear translocation of c-Myc, which binds to the *nfatc1* promoter along with bromodomain and extra-terminal (*BET*) proteins, which function epigenetically to induce *nfatc1* expression. OB precursors and synovial fibroblasts also express myostatin, which binds to and activates a heterodimeric complex of activin receptor 2B (ACVR2B) and ALK4 (also called ACVR1B) or ALK5 (also called TGFBR1) leading to Smad2-mediated NFATc1 nuclear translocation. OCs and OCPs express myostatin, which promotes OC formation by an autocrine mechanism that is less potent than the OB paracrine effect. In response to FGF21, hepatocytes express IGFBP1, which binds to the intergrin β1 receptor on OCPs and induces ERK phosphorylation and *nfatc1* expression.

the unfolded protein response, a cellular adaptive mechanism activated in response to accumulation of unfolded proteins in the ER.[32] This temporary induction of XBP1 may trigger the transient NF-κB/NFATc2-induced autoamplification of NFATc1 (Fig. 11.2) that occurs early in response to RANKL and suggests that the UPR has a limited role in the resorptive activity of mature OCs, which NFATc1 also regulates.[12] Basal and RANKL-induced NFATc1 expression is increased by insulin-like growth factor binding protein IGFBP1, a hormone released from the liver in response to FGF21, which is also produced by the liver and regulates glucose and lipid metabolism, but also induces bone loss.[33] IGFBP1 binds to the integrin β1 receptor on OCPs leading to increased ERK phosphorylation and NFATc1 activation during the later phase of OC differentiation, consistent with NFATc1's effects to induce expression of genes involved in OC activation (Fig. 11.3). IGFBP1 blockade prevented FGF21-induced bone resorption without affecting FGF21's positive insulin-mediated effects on metabolism, suggesting that IGFBP1 inhibitors could be used to prevent FGF21-induced bone loss.[33]

Nuclear receptors comprise a superfamily of transcription factors that also regulate OC formation (reviewed in Ref. 34). They are activated by numerous factors, including steroid hormones, retinoic acid, and lipid metabolites, but there are many with no known ligands, called orphan receptors. Nuclear receptors can activate or repress gene expression by recruiting coactivators or corepressors. They include estrogen, androgen, glucocorticosteroid, and vitamin D receptors, peroxisome proliferator-activated receptors (PPARs), estrogen-related receptors, liver X receptors, and retinoic acid receptors. Estrogen and androgen inhibit OC formation and promote their apoptosis, and glucocorticosteroids and vitamin D promote OC formation. However, their effects are complex, and there are conflicting data on the molecular mechanisms involved.[35] PPARs suppress differentiation of OBs from precursors and promote adipocyte differentiation, but they also promote OC formation. For example, PPARγ directly increases c-Fos expression in OCPs and PPARγ agonists, such as rosiglitazone, which are used to treat type-2 diabetes mellitus, induce bone loss, and increase fractures in patients after long-term use due to increased bone resorption and decreased bone formation. In contrast, liver X receptors inhibit OC formation by suppressing NF-κB and c-Fos activity, while retinoic acid receptors inhibit OC activation by binding to v-maf musculoaponeurotic fibrosarcoma oncogene family protein B (MafB) in mature OCs and impairing normal ruffled border formation.[36]

2.3 Costimulatory Signaling-Mediated Osteoclastogenesis

Costimulatory signaling is another important mechanism for inducing OC formation during inflammatory and immune responses by activating PLCγ/calcineurin-Ca^{2+} fluxes[8] (Fig. 11.2). It is activated in pathologic immune responses by immune complexes (ICs, which consist predominantly of polymerized IgGs) and ligand

binding to immunoglobulin-like receptors, such as triggering receptor expressed in myeloid cells-2 (TREM-2), OC-associated receptor (OSCAR), and PIR-A (Fc receptor common γ subunit; FcRγ).[8] Adaptor molecules, including DNAX-activating protein 12 (DAP12) and FcRγ are recruited to these receptors. Phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) within these adaptor proteins activates downstream signaling. Four FcγRs are expressed in mice, and of these FcγRIIB and FcγRIII are highly expressed in OCPs. In basal conditions in which IgG levels are low, FcγRIII binds to and sequesters FcγRIIB in OCPS, and it thus inhibits OSCAR- and FcRγ-mediated osteoclastogenesis. In bone destructive autoimmune diseases IC levels increase leading to down regulation of FcgRIII expression and an increase in the FcγRIIb/FcγRIII ratio, which favors osteoclastogenesis.[37] DAP12/FcRγ dKO mice are severely osteopetrotic because they do not form OCs due to impaired RANKL-induced NFATc1 activation, but DAP12$^{-/-}$ and FcRγ$^{-/-}$ mice have only mildly impaired OC formation.[8] Mice doubly deficient in Dap12 and αvβ3 integrin have moderate osteopetrosis, associated with normal osteoclastogenesis, but deficient attachment to bone matrix and thus reduced function.[38] Despite the essential function of DAP12/FcRγ, this signaling pathway cannot induce OC formation on its own.

The ligands for most costimulatory receptors have still to be identified, but OSCAR is activated in OCPs by specific motifs within collagen fibers in bone matrix that become exposed in resorption lacunae.[39] RANK- and costimulatory signaling-mediated PLCγ activation are linked directly in OCPs through Bruton and Tec TKs[40] (Fig. 11.2). These are members of the largest family of receptor-associated Tks, mutations of which are associated with immunodeficiencies, including Bruton's agammaglobulinemia, and with malignant lymphomas.[41] Costimulatory signaling likely enhances OC formation in inflammation-induced bone resorption to mediate what can be devastating effects on joints of patients with RA. T and B lymphocytes also regulate osteoclastogenesis in inflammatory arthritis by secreting RANKL. Th17 T cells, particularly those derived from Foxp3-Tcells, are the major subset of T cells responsible for osteoclastogenesis in RA.[42] IL-17 upregulates RANKL expression in synoviocytes and induces expression of TNF, IL-6, and IL-1 from immune cells, and these cytokines in turn upregulate RANKL expression by synoviocytes. TNF expression is also increased in OCPs in response to autoantibodies against citrullinated proteins, particularly citrullinated vimentin, which are raised in the serum of RA patients, bind to OC surfaces and increase osteoclastogenesis in vitro and bone resorption when injected into mice.[43] Thus, high levels of these autoantibodies in the serum of RA patients may predict more aggressive bone resorption.

2.4 RANKL/RANK Downstream Signaling

TNF superfamily receptors, including RANK, lack intrinsic protein kinase activity, and recruit adapter proteins, called TRAFs, to their cytoplasmic tails to mediate downstream signaling. RANK recruits TRAFs 1, 2, 3, 5, and 6 in OCPs[2,12] in response to RANKL, but of these only TRAF6 appears to have essential functions in OCPs. Two sets of TRAF6$^{-/-}$ mice have been generated; both are osteopetrotic, but interestingly one lacks OCs and the other is OC rich.[2,12] The difference in the OC phenotype remains to be explained definitively and may be related to different knockout strategies, but the findings indicate essential functions for TRAF6 in both OC formation and function. RANK/TRAF-mediated protein kinase signaling activates several pathways. Four of them mediate OC formation: NF-κB-inducing kinase/inhibitor of NF-κB kinase/NF-κB, JNK/activator protein-1 (AP-1)/c-Fos, c-myc, and PLCγ/calcineurin/NFATc1[2,12] (Figs. 11.2 and 11.3). Others mediate OC activation (Src and mitogen-activated protein kinase kinase 6 (MKK6)/p38/MITF) and survival (Src and ERK).[2,12] Several other adapter molecules bind to the intracytoplasmic domain of RANK to mediate signaling, including Grb-2-associated binder (Gab) protein 2, which recruits signaling molecules that contain Src homology-2 domains and mediates RANKL-induced OC differentiation.[44]

2.5 Regulation of Osteoclast Activation

OCPs are attracted to bone surfaces from the bloodstream by a variety of factors, including CXCL-12 and sphingosine-1 phosphate.[2] Once formed, OCs form tight, roughly circular, ring-like organelle-poor sealing zones onto the bone surface, which is exposed after lining cells covering quiescent bone surfaces retract their cytoplasm. These rings have actin filament-rich podosomes, which are surrounded by adhesion, adaptor, and signaling molecules.[45] Inside these attachment zones, secretory lysosomes fuse with the cytoplasmic membrane to form the finger-like processes of the ruffled border membrane in a process regulated by autophagy.[46] Attachment initiates OC activation mainly through the vitronectin receptor αVβ3 integrin[45] and requires expression of kindlin-3.[47] Kindlin-3$^{-/-}$ mice have severe osteopetrosis due to an OC adhesion defect,[47] and patients with kindlin-3 gene mutations also have osteopetrosis,[48] which is rescued by BM transplantation, consistent with an OC defect. However, studies using MSCs from these patients suggested that enhanced OB differentiation may also have contributed to the increased bone mass, a feature not seen in the kindlin-3$^{-/-}$ mice.[48]

β3 integrin- and Src-mediated signaling is essential for ruffled border and normal actin ring formation, and thus for bone resorption. β3 integrin recruits numerous

signaling proteins, including the TKs, Src, and Syk, and the guanine nucleotide exchange factor, Vav3, resulting in activation of the small GTPase, Rac1.[45] Src recruits a number of proteins to the vitronectin and other receptors and phosphorylates tyrosine residues on them. These include Pyk2, Syk, cortactin, and the ubiquitin ligase, c-Cbl.[49] Although Src mediates RANKL-induced survival signaling in vitro, OCs survive normally in $src^{-/-}$ mice in vivo, presumably because other members of the Src family substitute for it.

The ruffled border increases the cell surface area for the passage of H^+ and Cl^- ions, which form HCl outside the cell to dissolve the mineral, and proteolytic enzymes, particularly cathepsin K, to degrade the matrix.[2,45] H^+ ions are secreted through the V-type H^+ ATP6i proton pump complex and Cl^- flows through a chloride channel encoded by *ClCN7*. The brain-type cytoplasmic creatine kinase (Ckb) regulates ATP distribution and supply in OCs to mediate actin ring formation, RhoA GTPase activity, vacuolar ATPase function and thus osteoclastic resorption. Ckb siRNA or cyclocreatine, a Ckb inhibitor, suppress bone resorption in vitro, and $Ckb^{-/-}$mice are protected against Ovx-, LPS-, and IL-1-mediated bone resorption, suggesting that Ckb is a target for prevention of pathologic bone loss.[50] The organic and inorganic breakdown components of bone pass through the cytoplasm of OCs in vesicles and are secreted from the cytoplasm facing the BM cavity into the bloodstream in a process that also involves small GTPases.[2,45]

Most cases of osteopetrosis in humans occur because of mutations in genes involved in matrix demineralization and dissolution. These include: chloride channel 7 (ClCN7); T-cell, immune regulator 1 (TCIRG1), which encodes the α3 subunit of the H^+ ATPase; carbonic anhydrase II, which catalyzes hydration of CO_2 to H_2CO_3 to provide a source of H^+, cathepsin K, mutations of which result in pycnodysostosis; and Pleckstrin homology domain-containing family M member 1 (Plekhm1), which encodes for a vesicle-associated protein linked to small GTPase signaling.[2,45,51]

OCP fusion is an important component of OC activation, because high OC nuclear numbers correlate with more aggressive resorption. Cytoplasmic fusion occurs in resorption lacunae under the influence of a number of factors, including DC-STAMP,[52] OC-STAMP, CD9, Atp6v0d2, SHP2[53] protocadherin-7, vitamin E, strawberry notch homologue 2,[2,45] and Fra-2 through leukemia inhibitory factor (LIF)/LIF receptor signaling and hypoxia.[2,45] Atp6v0d2 is a subunit of V-ATPase, a component of the V-type H^+ ATP6i proton pump complex.[2,45] NFATc1 in conjunction with MITF and PU.1 regulates expression of a number of genes encoding some of these and other genes involved in several key OC functions, including NFATc1 itself, TRAP, cathepsin K, VATPase-d2, DC-STAMP, and OSCAR[2,16,45] (Fig. 11.2). Mutations in MITF result in reduced OC formation and fusion and osteopetrosis of varying severity.[2,45] MITF also has essential functions in melanocytes, and $MITF^{-/-}$ (gray lethal) mice have not only a "malignant" form of osteopetrosis, but also gray, rather than black hair because they have defective melanin production. MMP13 secreted by myeloma cells in BM promotes OCP fusion and bone resorption independent of its proteolytic activity.[54] NFATc1 also positively regulates expression of type 1 collagen (*Col1a1*) in conjunction with osterix (Fig. 11.2), a transcription factor which functions downstream of Runx2 to mediate OB differentiation.[2,45] Thus, it may be challenging to develop a bone resorption inhibitor whose function is based on preventing PLCγ/calcineurin activation of NFATc1 that does not also have detrimental effects on bone formation.[55]

2.6 Negative Regulation of Osteoclast Formation and Function

Calcitonin, a hormone released from C cells in the thyroid, was identified many decades ago as the first negative regulator of OC activity. Since then, many others have been identified and point to the high importance of negative regulation to limit both physiologic and pathologic bone resorption. For example, there are several transcriptional repressors expressed constitutively in OCPs to limit osteoclastogenesis that are downregulated by RANK signaling. These include Eos, MafB, inhibitors of differentiation/DNA binding (ids), which are induced by CCAAT-enhancer-binding protein β (C/EBPβ), Bcl6, B lymphocyte–induced maturation protein 1 (Blimp-1) and interferon regulatory factor-8.[56]

In unstimulated OCPs, Bcl6 is recruited to NFATc1, cathepsin K, and DC-STAMP promoters to inhibit OC formation and activation. RANK signaling induces loss of Bcl6 from these promoters and NFATc1 replaces it, suggesting that these factors have a reciprocal relationship in osteoclastogenesis.[57] Consistent with this, $Bcl6^{-/-}$ mice have accelerated osteoclastogenesis and severe osteoporosis. Bcl6 is a direct target of Blimp1, *Irf8*, and *Mafb*. Blimp1 deletion in OCs resulted in impaired osteoclastogenesis and osteopetrosis due to upregulation of Bcl6.[57] Signaling through CD11b and β2 integrins in OCPs treated with M-CSF limits osteoclastogenesis by suppressing RANK expression and inducing BCL6 binding to *NFATC1* and repressing its transcription. RANKL downregulates CD11b expression, but inflammatory mediators, including fibrinogen and complement split products also enhance CD11b signaling to limit inflammation-induced osteoclastogenesis.[58] Thus, RANKL/RANK induction of NFATc1 in OCPs not only promotes osteoclastogenesis directly, but also indirectly by repressing negative regulators.

RANKL also downregulates expression of inhibitory microRNAs in OCPs to induce OC formation.

These include miR-34a, which directly targets transforming growth factor-β-induced factor 2 (Tgif2). Tgif2 induces expression of NFATc1 and AP-1, and these in turn promote Tgif2 expression in an autocrine loop. Nanoparticle-delivered miR-34a prevented OVX-induced bone loss, while transgenic mice overexpressing miR-34a in OCs have reduced bone resorption and increased bone mass, similar to Tgif2$^{-/-}$ mice.[59] These findings suggest that miR-34a is a key OC suppressor and that its administration could reduce pathologic bone resorption. Several other miRNAs have been shown to regulate OC formation both positively and negatively.[59]

RANKL induction of osteoclastogenesis is associated with a shift to oxidative metabolism in OCPs and epigenetic regulation of gene expression, including methylation and histone modification. For example, increased oxidative metabolism results in production of S-adenosylmethionine from methionine and ATP in OCPs and increased expression of the de novo DNA methyltransferase, Dnmt3a, which stably silences gene expression in numerous cell types. Dnmt3a promotes OCP differentiation by methylating CpG motifs on *Irf8* and thus stably represses its expression. OC-specific *Dnmt3a*-deficient mice are osteopetrotic and protected from Ovx-induced bone loss. In addition, a Dnmt3a inhibitor reduced OC formation and bone loss after OVX, suggesting that Dnmt3a is a new therapeutic target for osteoporosis.[60]

Other negative regulatory factors include Leucine-rich-repeat containing G-protein-coupled receptor 4 (LGR4), a recently identified additional receptor for RANKL.[61] It competes with RANK and limits OC formation by activating $G\alpha_q$ and GSK3-β signaling, which suppresses expression and activity of NFATc1. Mice with global or OC-specific loss of *Lgr4* have increased OC numbers and size and osteoporosis. Soluble LGR4 extracellular domain inhibited OC formation in mice in vivo because it bound to RANKL, similar to OPG, and it reduced RANKL-induced bone loss in three mouse models of osteoporosis. Developmental endothelial locus-1 is a protein expressed by OCs that limits their formation and resorptive activity by inhibiting NFATc1 expression through a Mac-1 integrin-dependent mechanism. Locally injected human developmental endothelial locus-1 reduced bone resorption and bone loss in a nonhuman primate model of periodontal disease.[62] Discoidin domain receptor 2 (DDR2) is a receptor TK, which promotes OB differentiation. It also limits OC formation by facilitating binding of the coreceptors, Neuropilin-1 and PlexinA1, to form a DDR2-Nrp1-PlexinA1 complex, which blocks PlexinA1-mediated osteoclastogenesis. DDR2 also prevents PlexinA1 from interacting with TREM2 and DAP12.[63] Transmembrane protein 178 (Tmem178), a downstream target of PLCγ2, localizes to the ER and negatively regulates RANKL-induced Ca^{2+} fluxes to limit NFATc1 activation and OC formation particularly in inflammation-induced bone resorption.[64] In addition, Stat5 negatively regulates bone resorption in response to IL-3-induced activation of RANKL by limiting ERK signaling. This promotes expression of the MAPK phosphatases, dual specificity phosphatase 1 (Dusp1) and Dusp2, and promotes a cell-autonomous negative feedback loop in OCs.[65]

WNT16 is an OB-derived protein linked to low cortical bone mass in humans; it inhibits osteoclastogenesis directly by limiting OCP differentiation and indirectly by increasing OPG expression in OBs (Fig. 11.1). Wnt16$^{-/-}$ mice have low-cortical thickness associated with increased endosteal OC activity, but normal trabecular bone volume. Consequently the mice develop spontaneous tibial fractures, identifying Wnt16 as an important regulator of osteoclastogenesis and fracture susceptibility.[66] Wnt4a also limits OC formation by inhibiting TRAF6/Tak1/TAK1-binding protein 2–mediated NF-κB activation in OCPs (Fig. 11.1), and transgenic mice overexpressing Wnt4a in OBs are resistant to age- and TNF-induced inflammatory bone resorption, associated with increased bone formation in vivo, while recombinant Wnt4a prevented Ovx-induced bone loss, suggesting that positive targeting of Wnt4a signaling could prevent postmenopausal and age-related bone loss.[20]

Charged multivesicular body protein 5 (CHMP5), which regulates late endosomal trafficking and multivesicular body formation, cooperates with the Paget's disease genetic risk factor, valosin-containing protein (VCP/p97), to limit OC formation by downregulating ubiquitination of IκBα downstream of RANK in association with the deubiquitinating enzyme, USP15.[67] CHMP5 in OCs also negatively regulates bone formation by limiting expression of a number of OB stimulating factors from OCs. CHMP5$^{-/-}$ mice have increased bone remodeling and a Paget's disease-like bone phenotype.

There are other mechanisms to limit the effects of RANKL and TNF on OCs, particularly in inflammatory bone diseases. For example, in rheumatoid joints, T cells and synoviocytes express RANKL, which induces c-Fos activation in OCPs.[2,8,45] c-Fos not only induces OC formation, but also limits it by inducing expression of INFβ by OCPs.[68] T cells also secrete INFγ, which degrades TRAF6 in OCPs to enhance this inhibitory activity.[8] Although TNF induces osteoclastogenesis predominantly by inducing RANKL expression by accessory cells, it can also induce osteoclastogenesis directly.[69] Furthermore, it limits osteoclastogenesis directly in OCPs.[69] RANKL and TNF activate NF-κB RelA and p50 directly in the canonical NF-κB pathway in OCPs to promote osteoclastogenesis; they also induce expression of NF-κB p100, which acts as an inhibitory κB by binding to other NF-κB proteins.[69] Unlike RANKL, TNF does not efficiently process NF-κB p100 to p52 in the noncanonical NF-κB pathway and consequently p100 levels increase in the OCPs and

limit RANKL- and TNF-induced osteoclastogenesis.[69] This buildup of p100 is associated with a concomitant increase in TRAF3, which promotes degradation of NF-κB-inducing kinase to limit OCP differentiation. RANKL-induced TRAF3 degradation can be prevented by chloroquine, a drug still used in some parts of the world to reduce inflammation in RA and SLE. Chloroquine administration to mice prevented ovariectomy-induced bone loss, suggesting that it could be used as an antiresorptive drug.[70] This buildup of p100 and TRAF3 in OCPs in part explains why TNF does not induce OC formation when administered to either $RANKL^{-/-}$ or $RANK^{-/-}$ mice, but does in these mice when they are also deficient in p100.[69] TNF can also limit OC formation by inducing expression of interferon regulatory factor-8 and the Notch-induced RPB-Jκ.[56] TNF induces bone resorption by additional mechanisms. For example, it increases OCP proliferation in and egress from the BM into the bloodstream, making more of them available to be attracted by chemokines, such as SDF-1, expression of which TNF induces in inflamed joints.[2] Thus, there are several potential strategies that could be developed to limit TNF-induced bone destruction in RA.

Toll-like receptors are activated in monocytes at sites of inflammation by microbial products and this enhances their immune response functions. Toll-like receptors signaling in OCPs, as well as GM-CSF and IL-4 inhibit osteoclastogenesis at sites of inflammation by inducing the cells to shed the extracellular domain of c-fms.[56] IL-10 is an antiinflammatory cytokine, which helps to resolve inflammation in a variety of clinical settings. It inhibits expression of NFATc1, c-Fos, c-Jun, and TREM-2 in OCPs.[56] During costimulatory signaling, ITAM-bearing proteins typically interact with partnering proteins that contain an immunoreceptor tyrosine-based inhibitory motif,[56] some of which can promote and others limit immune responses and osteoclastogenesis. For example, the human inhibitory immunoglobulin-like receptor, LILRB, and the murine paired Ig-like receptor, PIR-B, recruit the SH2 domain-containing tyrosine phosphatase 1 (SHP-1) to negatively regulate osteoclastogenesis, while Ly49Q promotes it by competing with PIR-B for association with SHP-1).[71] CD80/86 complexes, which are expressed by antigen-presenting cells and bind to CD28 on T cells during costimulation, also negatively regulate OC formation through cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)–mediated activation of indoleamine 2,3-dioxygenase in OCPs, which degrades tryptophan and thus promotes OCP apoptosis.[72] CD80/86 dKO and $Ido^{-/-}$ mice are osteopenic due to increased osteoclastogenesis. Abatacept, a CTLA-4–immunoglobulin fusion protein, targets CD80/86 and inhibits osteoclastogenesis in humans.[72] Finally, interaction between ephrin B2, a protein expressed by OCPs, and its receptor, Eph 4, on OB precursors prevents c-Fos activation of NFATc1 to inhibit OCP differentiation[56] (Fig. 11.4).

2.7 Osteoclast Apoptosis

OCs are short-lived cells that die predominantly in the central and deeper parts of resorption lacunae as new OCs form at the cutting edges of the lacunae by addition of new mononuclear cells.[2] Cytokines, such as RANKL, M-CSF, TNF, IL-1, and VEGF-A, which induce OC formation and/or activation, also prevent their apoptosis through Rho family small G-protein Ras/Rac1/Erk and PI3 kinase/mTOR/S6K signaling.[2,45] Indeed, withdrawal of these cytokines from cultures leads to rapid induction

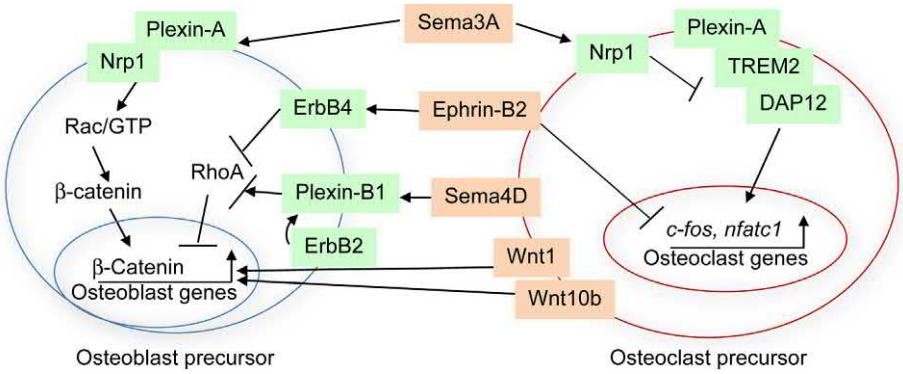

FIGURE 11.4 Signaling pathways regulated by OB–OC interacts. Sema3A is produced by OBs; its receptor, Plexin-A, is expressed on OB precursors and OCPs. Sema3A binds to Nrp1 on OB precursors and promotes β-catenin nuclear translocation via Rac/GTP to stimulate OB differentiation. Sema3A/Nrp1 signaling on OCPs disrupts Plexin 1/TREM2/DAP12-mediated (costimulatory) OC formation. Ephrin-B2 expressed on OCPs forward signals through its receptor, ErbB4, on OBs to enhance OB differentiation by causing Rho A inactivation. At the same time, ErbB4/Ephrin-B2-mediated reverse signaling into OCPs decreases OC formation by inhibiting *c-fos* and *nfactc1* transcription. Sema4D produced by OCs binds to its receptor, Plexin-B1, on OB precursors resulting in activation of ErbB2, which phosphorylates Plexin-B1. Sema4D binding to a Plexin-B1–ErbB2 receptor complex promotes bone formation by enhancing OB migration and differentiation. Wnts 1 and 10b are secreted by OCPs to promote OB precursor differentiation.

of OC apoptosis, in part mediated by reduced expression of Bcl-2, the principal member of the Bcl-2 family of antiapoptotic proteins.[73] Bcl-xL promotes OC survival in response to TNF, but not to RANKL.[2]

Mice with conditional deletion of Bcl-xL in OCs have increased OC apoptosis, but surprisingly they also have increased bone resorption apparently due to increased c-Src activity and expression of vitronectin and fibronectin by OCs, which increased integrin-mediated resorptive activity.[2,74] These findings suggest that Bcl-xL also inhibits OC resorptive activity. Bim is a proapoptotic BH3 homology-containing Bcl-2 family member whose expression is downregulated at the transcriptional level by IL-3-mediated Raf/Erk and/or PI3K/mTOR signaling. Bim is constitutively ubiquitylated and degraded on withdrawal of M-CSF, apparently by c-Cbl, an ubiquitin ligase, and $Bim^{-/-}$ mice have decreased OC activity, despite increased OC survival.[73] Thus, although in general, enhanced OC survival is associated with increased resorption and vice versa, these two activities can be uncoupled.

The earliest report of OC apoptosis was its induction by estrogen and tamoxifen in vitro through expression of TGFβ by BM cells cultured with 1,25 $(OH)_2$ Vitamin D_3.[75] Estrogen also increased OC apoptosis in vivo in mice suggesting that sex steroids limit bone resorption in part at least by limiting OC survival. More recent studies have reported that estrogen induces OC apoptosis by inducing Fas-ligand expression in OCs and OBs,[76] and that TGFβ can actually support OC survival directly through TAK1/MEK/AKT-mediated activation of NF-κB in RANKL/M-CSF-treated BM-derived OCs.[77] Thus, the effects of estrogen and TGFβ on OCs are complex. Nevertheless, administration of estrogen to sex-steroid-deficient rodents and women inhibits bone loss, in part by inducing OC apoptosis, which is also induced by high extracellular calcium concentrations that result from bone resorption.[78]

Bisphosphonates are the most widely prescribed anti-resorptive drugs. The mechanisms whereby they inhibit bone resorption have become much clearer in the past decade, but one of the earliest mechanisms identified was induction of OC apoptosis in vitro and in vivo[79] in part by caspase cleavage of Mst1 kinase. This effect is attenuated by TNF through upregulation of Bcl-xL in OCPs and OCs[80] and by glucocorticosteroids[81] by a mechanism that remains to be determined. Other studies suggest that for some nitrogen-containing bisphosphonates inhibition of OC activity, rather than promotion of apoptosis is the predominant inhibitory mechanism.[82] OPG can also induce OC apoptosis by binding to RANKL and preventing it from interacting with RANK to mediate survival and other signaling. However, OPG can also bind to TNF-related apoptosis-induced ligand, which induces OC apoptosis; one study has reported that OPG can reduce human OC apoptosis in vitro by inhibiting this mechanism.[83] Further studies are required to determine if this mechanism has a functional role in vivo.

3 OSTEOBLASTS

The term OB tends to be used rather loosely to describe a variety of cells in the lineage from committed precursors derived from MSCs to plump cells seen on bone surfaces laying down osteoid, but typically does not include osteocytes, which help to regulate and maintain bone homeostasis. OBs not only play critical roles in the skeleton, but they also influence a number of diverse processes, including hematopoiesis, cancer development and bone metastasis, and glucose and phosphate metabolism. OBs are controlled by numerous mechanisms, including key transcription factors and signaling pathways, epigenetic modification, and cell–cell communication, particularly with osteoclastic cells. Mutations related to some of these factors cause genetic disorders involving the skeleton. In this section, we describe the major factors and pathways that regulate OB formation and cellular communication under physiological conditions.

3.1 Transcription Factors and Signal Pathways

OB formation from MSCs is a two-step process involving lineage commitment of MSCs to osteogenic precursors followed by differentiation of pre-OBs and their maturation into OBs. Several critical signaling pathways and transcription factors are involved, including BMPs/Runx2, Wingless (Wnt)-β-catenin, and Notch. Mutations in these genes have been identified in human diseases associated with skeletal abnormalities, including craniofacial dysplasia, limb or joint deformities, and high and low bone mass. Given the important roles of these genes, it is not surprising that only patients with haploinsufficiency can survive, and that mutations are all autosomal-dominant.

3.1.1 BMP/RUNX2 Signaling

BMP signaling involves numerous ligands, receptors, and intracellular cascades. BMPs bind to their transmembrane serine-threonine kinase receptors to active canonical Smad dependent and noncanonical p38 mitogen-activated protein kinase signaling, resulting in expression of Runx2, an essential transcription factor for OB differentiation. The BMPs often have redundant roles in OB development. For example, BMP2, -4, and -7 regulate all stages of limb development, but single knockout mice have normal limb development.[84]

RUNX2 plays essential roles in bone formation by transactivating many genes required for OB differentiation. Runx2 is initially expressed in mesenchymal

condensations at E10.5, well before the appearance of chondroblasts or OBs at the very earliest stages of endochondral ossification.[85] It is essential for vascular invasion of the calcified hypertrophic cartilage anlagen by directly regulating transcription of VEGF-A.[86] RUNX2 levels are tightly controlled by multiple processes, including transcriptional regulation and protein modification, such as phosphorylation and ubiquitination. RUNX2 can directly link to the RNA polymerase II machinery via MED23, a member of the Mediator transcription complex. Mice deficient in Med23 in MSCs or OB precursors have a bone phenotype similar to that observed in *Runx2*$^{+/-}$ mice.[87] RUNX2 stabilizes *N*-α-acetyltransferase 10 (NAA10), a yeast protein whose function is poorly understood in mammals, which in turn inhibits OB differentiation by causing RUNX2 acetylation,[88] which also participates in RUNX2 regulation.

OB differentiation and maturation is associated with increased energy requirement, involving glucose metabolism and protein synthesis, both of which are regulated by key OB-positive regulators.[89,90,91] Glucose enters OBs through the glucose transporter (Glut1) whose expression in pre-OBs requires RUNX2. Crosstalk between Glut1 and RUNX2 determines the onset of OB differentiation and the extent of bone formation.[92] Furthermore, Wnt stimulates glutamine catabolism in pre-OBs, resulting in reduced intracellular glutamine levels, which activate a general control nonderepressible 2 (GCN2)-mediated integrated stress response to increase the translational capacity necessary for bone formation. Increased glutamine levels or depletion of GCN2 rescue the high bone mass phenotype of mice with increased Wnt signaling.[93] Manipulation of glutamine/GCN2 signaling may provide a valuable approach for normalizing deranged protein anabolism associated with human diseases.

Some of the genes encoding regulators of Runx2 are mutated in humans. For example, twist-1, a homeobox transcription factor, blocks Runx2 binding to DNA and individuals with *twist-1* haploinsufficiency suffer from Saethre–Chotzen syndrome,[94] an autosomal-dominant craniosynostosis with brachydactyly, soft tissue syndactyly and facial dysmorphism. Muscle segment homeobox homolog-2 (MSX2) is an upstream transcriptional activator of Runx2, and a single amino acid substitution in the *MSX2* gene causes Boston-type craniosynostosis,[94] a common developmental anomaly that causes abnormal skull shape due to premature closure of calvarial sutures. Moreover a genome-wide association study in 6000 Latin Americans implicated Runx2 in human facial variation.[95]

3.1.2 *Wingless (Wnt)-β-Catenin*

The Wnt family consists of a large number of secreted glycoproteins, which function by either paracrine or autocrine mechanisms. Wnt ligands bind to the 7-transmembrane domain-spanning Frizzled (Fzd) receptor and LRP5/6 coreceptors to stabilize β-catenin by preventing its phosphorylation. Unphosphorylated β-catenin translocates to nuclei, forms complexes with members of the LEF/TCF family of DNA-binding proteins, and regulates target gene expression. In the absence of a Wnt ligand, β-catenin forms a nonfunctional complex with adenomatous polyposis coli (APC), axin, glycogen synthase kinase 3, and casein kinase 1, which facilitate phosphorylation of β-catenin. Phosphorylated β-catenin undergoes ubiquitination and proteasomal degradation through its interaction with the F-box-containing E3-ligase, β-TrCP.[96]

Multiple Wnt ligands are expressed in bone during mouse limb development, including Wnts 2, 2b, 5b, 6, 7b, 9, 10a, 10b, 11, and 16.[97] Wnts 3a, 4, 5a, 7b, 10b, and 16 have been shown to bind to osteoblastic cells to regulate bone formation or osteoblastic control of osteoclastogenesis.[98,99] Interestingly, osteoclastic cells also secrete Wnt ligands, which regulate OB formation or function as a new mechanism for coupling bone formation to sites of new bone resorption. For example, mice with OC-specific deletion of TGF-β receptor (*Tgfbr2*$^{f/f}$*;cathepsin K-Cre*) develop osteoporosis due to reduced OB numbers without affects on OC numbers or activity. Wnt1 is secreted by OCs in response to TGF-β released from bone in resorption lacunae and stimulates OB precursor differentiation[100] (Fig. 11.4). In addition, TGF-β stimulates OCs to secrete Wnt10b, which promotes OB mineralization by a mechanism dependent on Smad2/3 activation and independent of TGF-β stimulation of protein kinase B or MAPK kinase[99] (Fig. 11.4). Wnt signaling also directly affects the cellular metabolism of OB-lineage cells by regulating aerobic glycolysis, glutamine catabolism, and fatty acid oxidation, which links bone anabolic signals and cellular bioenergetics.[101]

Frizzleds (Fzds) are G protein-coupled receptors to which Wnts bind and activate signaling.[102] Secreted Fzd-related proteins bind to and prevent Wnt signaling. Cell surface numbers of Fzds are controlled by a balance between E3 ligase ring finger protein 43- and zinc and ring finger 3-mediated ubiquitination[103] and deubiquitylase ubiquitin-specific protease-6-mediated deubiquitylation.[104] Roles for Fzds and their related proteins in bone came from analyses of several human skeletal disorders. Mutations in *Fzd4* are linked to familial exudative vitreo-retinopathy, an autosomal dominant disease characterized by progressive vision loss, reduced BMD and increased fracture risk in some patients.[105]

A critical role for LRP5 in bone was discovered following clinical reports that children with the autosomal recessive disorder, osteoporosis pseudoglioma, have a loss of function mutation in *Lrp5*, while patients with *Lrp5* gain of function mutations develop high bone mass associated with very strong, fracture-resistant bones.[106] These

clinical reports were confirmed in global *Lrp5*$^{-/-}$ mice, which have low bone mass associated with decreased Wnt signaling in OBs.[106] Mice deficient in Lrp5 specifically in OBs (*Lrp5*$^{f/f}$;*osteocalcin-Cre*) have reduced postnatal bone mass with increased body fat and decreased energy expenditure. Conversely, mice expressing a high bone mass mutant Lrp5 (G171V) allele are leaner with reduced plasma triglyceride and free fatty acid levels. Wnt-initiated signaling downstream of Lrp5, but not Lrp6, regulate expression of key enzymes required for fatty acid β-oxidation, suggesting that Wnt-Lrp5 signaling in OBs also regulates global energy homeostasis.[107] Mice carrying osteocyte-specific Lrp5 depletion (*Lrp5*$^{f/f}$;*DMP-1-Cre*) have smaller skeletons with reduced BMD, mineral apposition and bone formation rates in response to mechanical loading, indicating that Lrp5-mediated Wnt signaling in osteocytes significantly contributes to maintenance of biomechanical properties and bone mass.[108] Mice in which both Lrp5 and Lrp6 are conditionally deleted in chondrocytes and OBs (*Lrp5*$^{f/f}$;*Lrp6*$^{f/f}$;*collagen 2a1-Cre*) are embryonic-lethal with severe skeletal defects, while adult mice carrying Col2-cre-mediated deletions of Lrp5 and/or Lrp6 have low bone mass. These findings suggest that Lrp5 and Lrp6 have redundant functions in both embryonic skeletal development and regulation of adult bone mass.[109] These new findings emphasize the importance of LRP5 signaling in skeletal health and diseases associated with low bone mass.

LRP4 and LRP6 share a structure similar to the extracellular domain of Lrp5. *Lrp4*$^{-/-}$ mice die at birth because they can't breathe. OB-specific Lrp4 knockout (*Lrp4*$^{f/f}$;*osteocalcin-Cre*) mice develop high bone mass as they mature; by 8-months-old, they have increased bone formation, reduced bone resorption, and increased serum sclerostin levels. Loss of Lrp4 in OBs abolishes the inhibitory effect of sclerostin on Wnt/β-catenin signaling and OB differentiation because sclerostin requires Lrp4 to inhibit bone formation and impairs sclerostin induction of RANKL, leading to a lower RANKL/OPG ratio.[110] *Lrp4* mutations occur in patients with Cenani–Lenz syndrome (CLS), an autosomal-recessive disorder affecting distal limb development, associated with syndactyly and/or oligodactyly and kidney anomalies.[111] The CLS1 locus is mapped to chromosome 11p11.2-q13.1. Most CLS patients have splicing and missense mutations. Truncating mutations in *Lrp4* lead to a prenatal lethal form of CLS.[112] A novel splicing mutation in *APC* on chromosome 5q22 was reported in a CLS family with typical CLS features in addition to severe scoliosis. Thus, reduction of the negative regulator, APC, may increase availability of β-catenin by affecting its degradation, leading to a phenotypic similar to Lrp4 mutation.[113]

Lrp6$^{-/-}$ mice are perinatal lethal due to developmental abnormalities, including truncations of the axial skeleton, limb defects, and loss of the paraxial mesoderm.[114] OB-specific *Lrp6* knockout (*Lrp6*$^{f/f}$;*osteocalcin-Cre*) mice have significantly reduced bone mass, OBs and bone formation rates, but no change in OC and OB precursor numbers. They have more apoptotic OBs on bone surfaces and do not respond to the anabolic and anti-apoptotic effects of PTH.[115] Patients with a putative partial loss-of-function mutation in *Lrp6* are predisposed to early cardiovascular-related death, associated with high levels of plasma LDL and triglycerides, hypertension, diabetes, and osteoporosis.[116] Mutations, including missense, frame-shift, and splice-site mutation in *Lrp6* have also been detected in patients with tooth agenesis and/or orofacial clefting.[117,118]

Sclerostin is secreted by osteocytes and blocks Wnt signaling in part by binding to LRP5 and LRP6. Genetic deletion of sclerostin results in high bone mass due to increased bone formation in mice and humans.[119] Loss of function mutations and deletion of enhancer regions have been identified within the *SOST* gene encoding sclerostin in patients with sclerosteosis and Van Buchem disease.[120] Patients have generalized osteosclerosis, most prominent in the skull base, mandible, and long bones. Sclerosteosis is more severe than Van Buchem disease, and patients show additional features, including tall stature, syndactyly, and neurological complications due to cranial nerve compression. The difference in severity between these conditions may be explained by low levels of sclerostin in Van Buchem disease patients and undetectable serum sclerostin in sclerosteosis patients.[121]

3.1.3 Notch

In mammals, Notch comprises 4 (1–4) single-pass transmembrane receptors consisting of extracellular, transmembrane, and intracellular domains that determine cell fate following activation by 5 ligands (Jagged (Jag1) and Jag2, and Delta-like 1(Dll1), Dll3, and Dll4. Upon ligand binding, the Notch intracellular domain (NICD) is cleaved by γ-secretase and translocates to nuclei via its nuclear localization signal where it associates with and mediates removal of the RBP-Jκ transcriptional repressor to regulate expression of target genes, including Hes1 and Hey1, in the canonical pathway.[44] The C-terminus of the NICD contains a proline/glutamic acid/serine/threonine (PEST)-rich motif, which mediates its proteasomal degradation. Ubiquitination and deubiquitination regulate NICD stability through this PEST motif, contributing to tight regulation of Notch signaling in a spatial-temporal manner.[122]

Genetically modified mice with depletion of Notch[22] or γ-secretase[23] in the limb mesenchyme or OB precursors have markedly increased bone mass in adolescence followed by severe osteopenia as they aged. This led to a model that Notch signaling acts normally to maintain a pool of mesenchymal progenitors and inhibit OB precursor differentiation. Over-expression of NICD in

Col3.2-CreERT2 mice, in which the expression of the 3.2 kb *Col1a1* promoter is restricted to mature OBs and osteocytes, leads to increased bone mass and bone healing.[123] Conditional Notch activation in osteocytes increased bone formation in mice. Haploinsufficiency of the gamma-secretase, presenilin-1, which inhibits downstream Notch activation, inhibits terminal OB differentiation. Pharmacologic or genetic disruption of Notch or Jag 1 inhibits bone formation.[124] These studies suggest that Notch signaling is anabolic in mature OBs and osteocytes. However, RBP-Jκ depletion in mature OBs or chondrocytes did not affect fracture repair,[125] while depletion of RBP-Jκ in BM stromal cells resulted in fracture nonunion. These conflicting findings may reflect utilization of different Cre lines and the timing of Notch manipulation. In inflammation-induced bone loss, TNF activates Notch in MSCs, limiting OB differentiation.[126]

Mutations in *Jag1* are responsible for Alagille syndrome, an autosomal dominant disorder with defective development of the liver, heart, eyes, and bones. Skeletal abnormalities include "butterfly" vertebrae, narrower lumbar spine interpedicular spaces, and craniofacial deformities.[127] Mutations in *Notch 2* were identified by whole-exon sequencing of DNA from patients with Hajdu–Cheney syndrome[128] in which there is progressive focal bone destruction and osteoporosis, craniofacial anomalies, and renal cysts.[129] These generated a mature form of Notch 2 protein that contains a disrupted or absent proteolytic PEST sequence endowing it with an increased half-life and persistence of Notch intracellular signaling.[130] Exon sequencing revealed a compound heterozygous *Jag1/Notch 2* mutation in a patient with Alagille syndrome.[131]

OB lineage cells affect the homing and long-term repopulating of hematopoietic stem cells (HSCs), HSC mobilization, and lineage determination[132] and in the induction of leukaemogenesis. Mice carrying an activating mutation of β-catenin in OBs ($Catnb^{+/lox(ex3)}$;$\alpha_1(I)Col\text{-}Cre$) develop acute myeloid leukemia. β-catenin stimulates expression of Jag1 in OBs, which activates Notch signaling in HSCs to induce the leukemia. Increased β-catenin and Notch signaling was reported in OBs and hematopoietic cells, respectively, in 38% of patients with myelodysplastic syndromes or acute myeloid leukemia.[133] These new findings suggest that targeting Notch could increase bone mass in osteoporotic patients or at sites of osteolytic bone metastases. However, this likely will be challenging, given that the effects of Notch vary depending upon the stage of OB differentiation. Thus, Notch inhibitors might be most effective with fewer side-effects in patients with activated Notch signaling.

3.2 Epigenetic Modifications

Epigenetic regulation refers to functionally relevant modifications to the genome that do not involve changes in nucleotide sequence and act by modifying the accessibility of genes to transcription factors and other modulators. Epigenetic mechanisms play a central role in the promotion of appropriate transcriptional signaling during skeletal development and homeostasis. Epigenetic modifications include DNA methylation, histone modifications and micro-RNA and are carried out by complicated networks of epigenetic modifiers.

3.2.1 DNA Methylation

DNA methylation alters cytosines in DNA without changing the genomic DNA sequence and is modified by environmental factors and gene polymorphisms, linking environmental influences to disease pathogenesis. Most major signaling pathways in OBs are affected by DNA methylation of promoter regions of osteogenic genes.[134] OB differentiation from MSCs or precursor cells is associated with decreased DNA methylation in the promoters of homeobox 5 *(Dlx5)* and Osterix (*Osx)*, and increased expression of alkaline phosphatase, DLX5, OSX, and RUNX2.[135] BMP-2 causes hypomethylation of the *Dlx5* promoter, thereby inducing *Dlx5* expression and OB differentiation.[136] DNA methylation of the *SOST* promoter occurs close to binding sites for osteogenic transcription factors, including RUNX2 and promotes OB differentiation.[137,138] *SOST* proximal promoter DNA methylation is inversely associated with *SOST* levels in iliac bone samples.[139] MSCs cultured under mechanical loading conditions or in OB-inducing medium have decreased methylation at the *osteopontin* promoter as well as increased Osteopontin expression.[140]

3.2.2 Histone Modification

Histones are the major protein components of chromatin and act as coils around which DNA winds. There are five major histone families: H1/H5, H2A, H2B, H3, and H4. Histone modification includes methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, citrullination, polyadenylation, and glycosylation, of which histone acetylation and methylation have been studied most. Histone modifications typically promote OB differentiation. For example, acetylation of histones H3 and H4 increases accessibility of the *osteocalcin* promoter to Runx2 and other osteo-inductive transcription factors. Histone acetylation is carried out by histone acetyltransferases and deacetylases (HDACs), while histone methylation requires methyltransferases and demethylase.[141] Among these, the most widely studied in bone are the HDACs, which affect OB commitment and differentiation by regulating bone-related genes[142] and modifying intracellular signaling pathways.[143] Drugs targeting epigenetic modulators have been tested in various disease models, and Vorinostat, an FDA-approved histone deacetylase inhibitor, is used to treat cancer patients. However, nonspecificity of epigenetic-targeted drugs

and potential adverse effects will likely limit their use in chronic diseases, such as osteoporosis. Thus, understanding of which epigenetic modulators are expressed and function in bone cells may help to design more bone-specific inhibitors.

Oculo-facial-cardio-dental syndrome (OFCD), an X-linked dominant disorder that mainly affects development of the eyes, facial features, heart, and teeth, occasionally includes syndactyly of toes 2–3. OFCD is due to mutations of the BCL-6 corepressor, which normally catalyzes demethylation of Histone H3 lysine 36, but fails to do so when mutated. The resultant methylation reduces binding of BCL-6 to the *AP-2α* promoter, leading to uncontrolled AP-2α expression and osteo/dentinogenic differentiation of MSCs in OFCD.[144]

3.2.3 Micro-RNA

Micro-RNAs (miRNAs) are small, 16-25bp, noncoding RNA sequences that silence RNA and regulate post-transcriptional gene expression by binding to the 3′- untranslated regions of target mRNA. Hundreds of miRNAs are involved in bone regeneration and many regulate the expression of key OB regulators.[145] For example, MR-204, miR-23a, miR-30c, and miR-34c target Runx2[146] and miR-214, miR-145, and miR-31 target *Osx*.[147] miR-135 attenuates *Smad5* expression.[148] miR-335 enhances osteogenesis indirectly by decreasing expression of *dkk1*, a specific inhibitor of Wnt/β-catenin signaling.[149] MiR-210 and miR-34c regulate TGF-β/activin[150] and Notch signaling.[151] Elevated miR-214 levels correlate negatively with the degree of bone formation in fracture specimens from aged patients. Blockade of osteoblastic miR-214 by miR-214 antagomir prevents ovariectomy- or hindlimb unloading-induced bone loss, suggesting that miR-214 may be a therapeutic target for treating osteoporosis.[152] miR-188 is highly expressed in BM MSCs from aged mice and humans. Overexpression of miR-188 in Osx-expressing osteoprogenitors reduces OB differentiation and promotes adipogenesis by directly targeting HDAC9 and mTOR signaling, suggesting that miR-188 may be a key regulator of age-related switching from osteogenesis to adipogenesis.[153]

3.3 Regulation of Osteoblast/Osteoclast Communication

Normal bone remodeling is maintained by close communication between osteoclastic and osteoblastic cells. It is initiated by OC-mediated bone resorption, releasing coupling factors from bone matrix, including TGFβ and IGF-1, which recruit OBs to bone resorption sites. TGFβ is activated by the acidic environment generated by OCs in resorption lacunae and appears to induce migration of OB precursors to resorbing surfaces,[154] a role that also has been attributed to S1P and BMP6 expressed by OCs.[155] Ephrins and Semaphorins are other "OC–OB communicating factors" that regulate bone formation (Fig. 11.4). These were first identified as axon-guidance molecules that regulate communication between neurons and axons during development of the nervous system.[156] They are also expressed in endothelial and T cells and outside the nervous system where they control cell migration, immune responses, tissue development, and angiogenesis.[157,158]

3.3.1 Ephrins

Ephs (erythropoietin-producing hepatocyte kinases) receptors and their interacting ligands, ephrins (Eph receptor-interacting proteins) are cell surface molecules that mediate both forward and reverse (thus, bidirectional) cellular responses. Fourteen Eph receptors and eight ephrin ligands have been identified in humans. OCs express ephrinsB1 and B2, and OBs express EphB receptors, particularly EphB4. Interaction of ephrinB2 on OCs with EphB4 on OBs leads to ephrinB2-mediated reverse signaling in OCPs to decrease c-Fos and NFATc1 expression and inhibit osteoclastogenesis, and forward signaling to enhance OB differentiation through EphB4-mediated Rho A inactivation[159] (Fig. 11.4). OBs also express ephrin ligands, and their expression is increased by PTH or PTHrP, which may in part mediate the bone anabolic effects of these hormones.[160] Mice carrying OB specific *EfnB2* deletion (*Efnb2$^{f/f}$;Osx1-Cre*) have delayed bone mineralization and a 50% reduction in OB differentiation in response to PTH administration. These defects are associated with significantly lower mRNA levels of late OB differentiation markers and greater levels of OB and osteocyte apoptosis, indicating an antiapoptotic role of ephrinB2 signaling in maintaining OB differentiation and bone strength.[161]

Mutations in *ephrinB1* have been reported in patients with craniofrontonasal syndrome, an X-linked developmental disorder in which affected females have multiple skeletal malformations, including asymmetry of craniofacial structures and abnormalities of the thorax.[162] Ephrin-Eph receptor signaling also appears to be involved in cancer-induced bone loss. For example, ephrinA1 and Eph A1 mRNA expression is decreased in bones from patients with metastatic prostate cancer[163] and giant cell tumor of bone.[163] Myeloma cells appear to downregulate Eph4 expression in osteoprogenitors, which could account in part for the reduced bone formation seen typically at involved sites.[163] Thus, strategies to enhance ephrin and Eph4 signaling in osteoclastic and osteoblastic cells, respectively, could enhance bone formation and inhibit bone resorption.

3.3.2 Semaphorins

Semaphorins are axonal guidance repellents that induce growth cone collapse during neuronal development and comprise a large family of secreted and

membrane-bound glycoproteins characterized by a conserved amino-terminal "Sema" domain. Semaphorins are implicated in communication between different bone cell types by relaying autocrine or paracrine signals.[164] Semaphorin 4D (Sema4D) is selectively expressed by OCs and binds to its receptor, Plexin-B1, on OBs, resulting in activation and autophosphorylation of ErbB2, which phosphorylates Plexin-B1 (Fig. 11.4). RhoA associates with Plexin-B1, and binding of Sema4D to the Plexin-B1–ErbB2 receptor complex alters the activity of RhoA-associated protein kinase, affecting two aspects of OB functions: RhoA/cadherin-11-mediated spontaneous migration and IRS-1/IGF-1-mediated differentiation.[165] *Sema4d*$^{-/-}$ mice have high bone mass associated with increased OB numbers and bone formation, but normal OC numbers.[165] Bone scaffolds loaded with siRNA for Sema4D enhances bone fracture repair in ovariectomized rats.[166]

Sema3A is predominantly expressed by OBs, while its receptor, Plexin-A1, is expressed both on OCPs and OB lineage cells. Plexin-A1 promotes OC differentiation by activating ITAM signaling via formation of a Plexin-A1–TREM-2–DAP12 complex.[167] Binding of Sema3A to Neuropilin-1 (Nrp1) reduces OCP differentiation by sequestering Plexin-A1 from TREM2 and suppressing ITAM signaling (Fig. 11.4). Sema3A also inhibits OCP migration by suppressing RhoA activation. In contrast, Sema3A stimulates OB differentiation through activation of the canonical Wnt/β-catenin pathway. Binding of Sema3A to Nrp1 results in activation of Rac1 through a Rac-specific GEF FARP2, which enhances nuclear accumulation of β-catenin (Fig. 11.4). *Sema3a*$^{-/-}$ mice are osteoporotic due to increased bone resorption accompanied by decreased bone formation. Thus, bone mass is controlled by semaphorin signaling between OBs and OCs.

Given the roles of Ephrin and Semaphorin signaling in bone cells, it will be very important to determine if they interact with one another and if there are defects in them that lead to bone diseases in humans. Interestingly, a pathway-based genome-wide association analysis in a cohort of 1000 unrelated US Caucasians revealed that among 76 pathways studied EphrinA-EphR associated most significantly with variability in femoral neck bone geometry and biomechanical strength.[168]

The importance of these new findings may go beyond OC/OB interactions. Ephrins and semaphorins are expressed by other cells, including vascular endothelial cells, T cells, and certain cancer cells; interactions between these and bone cells could contribute to changes in bone mass in pathological conditions where the functions of these cell types are altered. Interruption of the interactions between ephrins or semaphorins and their receptors might be a new target for the development of therapies to induce anabolic responses in bone in a variety of conditions.

4 CARTILAGE

Cartilage is comprised primarily of matrix (collagens and proteoglycans) that encases sparse populations of chondrocytes, which generate and maintain the matrix. There are various types of cartilage, which include the least abundant elastic cartilage (found in the pinna of the ear, trachea, epiglottis), fibrocartilage (primarily forms as a response to injury), and the most abundant, hyaline cartilage. Hyaline cartilage is also found in the trachea and bronchial tubes, in the articular surfaces of diarthrodial joints, and during development in craniofacial structures and growth plates, which drive limb lengthening and bone growth pre- and postnatally. Chondrogenesis and endochondral bone formation are not restricted to the developing skeleton; indeed, chondrocyte proliferation, maturation, hypertrophy, and terminal differentiation are reinitiated at sites of fracture repair. Additionally, diseases of cartilage, such as osteoarthritis (OA) also have significant effects on the differentiation of chondrocytes and maintenance of articular cartilage. Consequently, the cellular and molecular mechanisms that regulate chondrogenesis and chondrocyte differentiation have been studied intensively. This section describes the processes of chondrogenesis and chondrocyte differentiation and highlights some of the critical regulators and molecular targets.

4.1 Chondrocyte Formation

Chondrogenesis is required for the formation of the mineralized skeleton in vertebrates. This process begins with the aggregation and condensation of loose mesenchymal tissue to form anlagen comprised of cells that actively express various extracellular matrix (ECM) and cell adhesion molecules, importantly aggrecan (Agc1) and the IIa splice form of the α1 chain of type 2 collagen (Col2α1-IIa).[169,170] Regulation of temporal and spatial ECM secretion and the coordinated parallel steps of differentiation rely on the action of numerous morphogens/growth factors, receptors, and transcription factors. The earliest events in the mesenchyme and sclerotome that participate in anlagen formation are initially driven by the morphogen Sonic Hedgehog (Shh) via its transmembrane receptors Patched-1 and -2 (Ptch1,2), leading to recruitment of another transmembrane protein Smoothened (Smo) and activation of Gli transcription factors.[171] Activation of this signaling system leads to the induction of various targets, including the master regulator of chondrogenesis, Sox9,[172] and Nkx3-2, which maintains Sox9 expression in condensing cells.[173]

Once the early developmental signals downstream of Shh initiate Sox9 upregulation, a series of other signaling pathways contribute to its tight regulation and thus the progression of chondrogenesis. Deceleration of

chondroprogenitor entry into the chondrocyte lineage is in part restricted by Notch inhibition of Sox9 via the transcription factor, RBP-Jκ, and the downstream activation of HES1 and HES5, with chondrogenesis ensuing only following reduced signaling in this pathway.[174,175] Once Notch repression is overcome, several key pathways further drive Sox9 upregulation, expansion of chondrogenesis and entry into the chondrocyte hypertrophic program. These include FGFs,[176–178] hypoxic conditions and the concomitant upregulation of Hypoxia-inducible factor 1α (HIF-1α),[179] and TGFβ.[180] BMPs also possess potent chondrogenic activity, and consistent with this, blockade of signaling by the decoy receptor, Noggin, inhibits cartilage formation.[181] Overall, these signaling pathways and others work in concert with each other to modulate progenitor commitment to the chondrocyte lineage. The full integration of signaling modulators that drive chondrogenesis, and the subsequent maturation of chondrocytes during endochondral ossification summarized below was reviewed recently.[169]

4.2 Endochondral Ossification

As anlagen elongate during endochondral ossification, growth plates form near each end of the element, and periarticular chondrocytes near the distal ends develop a spherical shape, continue to show Sox9 upregulation, and progressively upregulate chondrocyte lineage markers, including the IIb splice form of Col2a1 and Agc1,[169,170] as well as specific downstream targets of the Indian hedgehog (Ihh) signaling pathway.[182] As chondrocytes proliferate in the centers of anlagen and undergo the early steps of maturation, they flatten and form columns parallel to the axis of longitudinal growth. Flat columnar chondrocytes, the most proliferative cells in the growing cartilage elements, express low levels of Runx2 and Osterix (Osx) and high levels of Fgfr3, Nkx3.2, and Ptc1.[169] Prehypertrophic chondrocytes enlarge slightly and initiate expression of Ihh,[183] PTHrP and its receptor (Pth1r),[184,185] and other cooperative transcription factors/cofactors, including Mef2c/2d,[186] Foxa2,[187] HDACs,[188] and Zfp521.[189] Overall, interactions among these signaling pathways dictate the pace of cell proliferation and entry into hypertrophy. Ultimately, due to progressive domination of Ihh signaling and the influence of other factors that drive chondrocyte maturation, including FGFs, BMPs, and Wnt activators of β-catenin signaling,[169] these columnar cells begin the process of hypertrophy and withdraw from the cell cycle.

As hypertrophy ensues, chondrocytes increase expression of Osx[190,191] and the master transcriptional regulator of skeletal mineralization, Runx2,[192–194] which drive enlargement of the cells and expression of molecules that facilitate matrix mineralization, including Col10a1, alkaline phosphatase, MMP9&13, osteopontin (Spp1), and osteocalcin.[169,170] Terminal maturation is triggered by IGF-1,[195] and mature chondrocytes located in the center of the anlagen produce high levels of VEGFA, which is thought to promote vascularization of the cartilage as these cells undergo apoptosis.[196] Only the most terminal hypertrophic chondrocytes express MMP13, an enzyme that controls cartilage matrix degradation, facilitates vascular invasion, and is required for creation of the marrow space.[191]

During early postnatal development, epiphyseal chondrocytes (immature chondrocytes in the epiphyseal centers at the ends of long bones) undergo maturation similar to the chondrocyte differentiation that occurs in the middle of anlagen. These cells differentiate, hypertrophy, undergo apoptosis, and are replaced by invading vasculature and OBs creating a secondary ossification center, which separates the only two areas of remaining cartilage within individual long bones of the growing skeleton: articular and mature growth plate cartilage.[197] When chondrogenesis occurs in adults, such as during fracture repair, secondary centers, or novel limbs are not formed, but repair progresses essentially the same way and hypertrophic chondrocytes are ultimately required for analogous purposes (initiation of mineralization and induction of vascular invasion).

Disruption of this orderly process of chondrogenesis and chondrocyte maturation has a significant impact on formation and growth of the skeleton. More than 380 conditions are associated with gene mutations in key signaling pathways that modulate the chondrogenic lineage and differentiation. For example, heterozygous mutations in Runx2 in humans cause cleidocranial dysplasia ,[198] consistent with this factor's central role in skeletal mineralization. Either inhibition or overactivation of signaling in the PTHrP pathway leads to disruption of orderly chondrocyte hypertrophy and dwarfism.[199,200] In fact, activating or inactivating PTHrP receptor mutations lead to a series of growth disorders, including Blomstrand's lethal chondrodysplasia, enchondromatosis, and Jansen metaphyseal chondrodysplasia.[201] An inactivating mutation in intestinal cell kinase (ICK) impairs chondrocyte Ihh signaling and causes short rib-polydactyly syndrome,[202] and inactivating mutations of FGF receptors in humans cause multiple growth defects, including Pfeiffer syndrome (FGFR1[203]), Apert Syndrome (FGFR2[204]) and craniosynostosis, achondroplasia, and hypochondroplasia (FGFR3[205–207]). Multiple hereditary exostoses is associated with an inactivating mutation of EXT1 and EXT2, leading to defective heparin sulfate production by chondroprogenitors and chondrocytes leading to cartilage-capped bony outgrowths.[208] Finally, metachondromatosis, an autosomal dominant tumor syndrome featuring multiple exostoses and enchondromas,[209,210] is associated with activation mutations of PTPN11[211,212] and downstream activation of Ihh

and PTHrP signaling in chondroprogenitors.[213] A comprehensive list of other less common genetic diseases in humans that are associated with disrupted chondrocyte function, signaling, or metabolism has been compiled and described.[214]

4.3 Endochondral Ossification in the Adult Skeleton

Healing of unstabilized skeletal fractures is supported by chondrogenesis and endochondral ossification, which is initiated by an early inflammatory response to injury.[215] Following cortical bone fracture or osteotomy, local progenitor cells residing in the periosteum, BM or other niches are sensitized, enabling them to respond to biological or biophysical stimuli produced within the local injury milieu.[216] In contrast, stabilized fractures heal by intramembranous bone formation with virtually no cartilage formation and direct osteogenic responses in the periosteal layer.[217] While the source of cells that contribute to cortical bone repair generally arise from multiple pools, in either endochondral or intramembranous healing, the periosteum is a major source of either chondro- or osteo-progenitors[218]). Data suggest that periosteum-initiated bone repair might be analogous to fetal limb bud development,[219] including reliance of the process on activation of essential pathways in limb development discussed above, including BMPs/TGFβ, PTHrP, Ihh, and Wnt signaling. Thus skeletal dysplasias that result from defects in these signaling networks likely have critical implications in successful fracture repair. Moreover, given the role of these pathways in the healing process, therapeutics to address delayed healing or fracture nonunions have been or are under development. These include targeting of the Pth1r with teriparatide,[220,221] activation of the Wnt/β-catenin pathway with antibodies that inhibit the decoy receptor-binding activities of Sclerostin[222] and Dickkopf (DKK) family members,[223] and BMP receptor agonists.[224]

4.4 Formation of Articular Cartilage

Articular cartilage development begins during embryogenesis at sites of synovial joint formation through a sequential series of steps that include patterning of the joint site, interzone formation, cavitation, and morphogenesis.[225,226] Articular chondrocytes are formed from interzone cells and, unlike human growth plate chondrocytes, which are removed completely following adolescent growth, they persist; although the mechanisms that maintain them are largely unknown. Adult articular cartilage is maintained as four distinct cellular zones from the surface to the underlying bone: superficial, intermediate, radial, and calcified cartilage. The superficial zone consists of 1–2 cell layers of flattened chondrocytes expressing Proteoglycan 4 (Prg4) (also known as superficial zone protein or lubricin), Sox9, Col2a1(IIb), Agc1, Tnc and low levels of cartilage intermediate layer protein (Cilp).[226] Chondrocytes of the intermediate zone are round and express many of the same molecules as the superficial zone except for Prg4, although they have higher levels of Cilp. Radial and calcified cartilage zone chondrocytes express markers of chondrocyte differentiation and hypertrophy, including Col10a1.[226] Each zone is maintained throughout adulthood unless stress-related injury, inflammation, or genetic defects lead to loss of the signals required to maintain or inhibit excessive differentiation of chondrocytes. These pathways that decelerate or prevent chondrocyte hypertrophy are disrupted or impaired in OA,[227] leading to progressive loss of biomechanically appropriate articular cartilage matrix.

4.5 Cartilage Degeneration

OA is the most common form of arthritis, characterized by dysfunction of articular chondrocytes, articular cartilage and meniscal degradation, periarticular bone formation (osteophytes) and often enhanced bone density below the articular cartilage surface (subchondral sclerosis).[227] While the etiology of OA is not fully understood, it is generally held that biochemical, metabolic, genetic, and trauma-related factors participate in the progression of overall joint degeneration.[227] In the early stages of disease, synovial cells produce catabolic cytokines that initially induce transient articular chondrocyte proliferation and increased matrix synthesis (Col2a1, Agc1).[228,229] However, chronic low level production of these cytokines, which include TNF, IL-1, IL-17, and -18, and PGE_2 lead to enhanced synthesis of collagenases (MMP-1, -8, -9, -13) and aggrecanases (ADAMTS4 and 5), which drive matrix degradation, and progressively erode the articular surface.[230,231] This is considered to be an initiating step in the disease process and is associated with inappropriate expression of genetic and morphologic markers of endochondral ossification by articular chondrocytes, including Runx2, Col10a1, and increased apoptosis.[232]

Several pathogenic mechanisms have been associated with the initiation and progression of OA and matrix catabolism characterized by decreased collagen networking, proteoglycan loss and reduced cartilage stiffness. These include mechanical overloading of cartilage, such as high intensity exercise, abnormally large static or sudden loading, and injury to cartilage or surrounding structures (such as the shock-absorbing meniscus in the knee), all of which increase the risk of developing OA between 6- and 20-fold in humans[233] and are associated with increased cytokine production. Specifically, IL-1 and TNF induce expression of catabolic proteins, such as MMPs and aggrecanases, and prostaglandin and nitric

oxide, which promote chondrocyte injury and apoptosis.[231,234] Articular chondrocytes in degenerating cartilage also have more reactive oxygen species, including superoxide anion and hydrogen peroxide, which also likely contribute to the pathogenesis of OA by enhancing production of MMPs and cytokines.[235]

While there are several transgenic/knockout mouse models of OA that identify important roles for key matrix molecules, signaling networks, enzymes and cytokines in the disease process, perturbation of genes involved with the regulation of chondrocyte maturation are particularly pathogenic, based on their influence over the hypertrophic program in articular chondrocytes.[236] In other words, with a singular focus on the chondrocyte, the genetic basis of disease progression may be related to defects that are specific to signaling pathways involved with controlling articular chondrocyte maturation. For example, one early study established that increased β-catenin signaling, caused by an inactivating polymorphism of the Wnt decoy receptor, FrzB, is associated with increased incidence of hip OA in women.[237] This study has prompted growth of the field's focus on the genetic basis of OA, with work over the past fifteen years identifying other OA-associated polymorphisms, with a pool of more than 50 disease-associated SNPs identified.[238] Included are various IGF signaling modifiers, VEGF, interleukins 6, 8, 16, and 17, Insulin Receptor, TGFβ1 and associated Smad signaling molecules, and COX2. Some of these mutations have been validated in studies using mouse genetics, and development of cartilage-targeted therapeutic strategies may hinge directly on our understanding of these signaling network contributions to disease.

5 CONCLUSIONS

Understanding of the mechanisms that regulate skeletal development and bone remodeling and repair has improved considerably over the past 25 years as a result of advances in molecular biology and genetics and development of techniques that permit deletion or overexpression of genes in animals. Many inherited skeletal diseases, such as osteopetrosis, juvenile osteoporosis, sclerosteosis, and cleidocranial dysplasia, have been attributed to mutations identified in specific genes that regulate OC or OB formation or functions or cartilage mineralization following seminal studies in knockout mice and human genetics. Initial studies identifying an essential role for RANKL expressed by osteoblastic stromal cells in OC formation and activation have been followed many others showing that chondrocytes, osteocytes, lymphocytes and synoviocytes are also involved and that RANKL also activates many negative pathways to limit bone destruction. There are complicated interactions between osteoclastic and osteoblastic cells that positively and negatively influence one another's formation and functions involving Wnt, Notch, and networking pathways that have given more insights into how bone remodeling is regulated locally. However, these findings have also raised many questions about how systemic hormones, growth factors, and cytokines influence these pathways and cellular interactions locally along with mechanical forces to regulate bone mass in normal and disease states. Future studies are required to determine precisely which osteoclastic and osteoblastic cells interact in remodeling units to ensure that they are filled completely during normal remodeling and how these interactions are disrupted in pathologic processes that lead to pathologic bone loss.

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CHAPTER

12

Overview of Bone Structure and Strength

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1 INTRODUCTION

Fracture associated with osteoporosis represent an immense and increasing public health issue. Based on current demographic trends, the number of fractures and their associated costs are projected to double or triple in the near future.[1] Indeed, by 2025, annual costs attributable to osteoporosis and fractures are expected to exceed USD 25 billion in the United States alone.[2] Most importantly, the consequences of these fractures are enormous, as those who suffer fractures experience increased mortality rates, chronic pain and disability, and a decreased quality of life.[3]

Strategies designed to prevent fractures require a sound understanding of their etiology. From an engineering viewpoint, fractures of any type are due to a structural failure of the bone. This failure occurs when the forces applied to the bone exceed its load-bearing capacity. Engineering principles dictate that the load-bearing capacity of a bone depends on the bone's morphology (e.g., its size, shape, distribution of bone mass, and microarchitecture), the intrinsic mechanical properties of the bone matrix, and the rate and direction of loads applied to the bone. Thus it is clear that factors related both to the forces applied to the bone, as well as to its load-bearing capacity are important determinants of fracture risk. Thus, identification of the genetic determinants of fracture risk are not only complicated by the unpredictable role played by skeletal loading (e.g., falls), but also by the fact that several factors contribute to bone strength. Whereas areal bone mineral density (BMD) is associated with whole-bone strength and strongly associated with fracture risk. Newer imaging modalities may allow further investigation of the genetic contributions to bone morphology and microarchitecture.[4–6]

In this chapter clinical and laboratory studies demonstrating age-related differences in bone morphology and the contribution of bone structure to bone strength are reviewed. First, the basic concepts related to the biomechanics of bone, including a summary of the factors that determine the material and structural behavior of bone, are presented, and second, the specific age-related changes in bone structure and their impact on skeletal fragility are discussed.

2 BONE BIOMECHANICS AND THE DETERMINANTS OF WHOLE-BONE STRENGTH

The ability of a bone to resist fracture (or whole-bone strength) depends on the amount of bone (i.e., mass), the spatial distribution of the bone mass (i.e., shape, ratio of cortical to trabecular bone, and microarchitecture), and the intrinsic properties of the bone matrix (Fig. 12.1). Bone remodeling, specifically the *balance* between formation and resorption, is the biologic process that mediates changes in the traits that influence bone strength. Thus, diseases, conditions, and drugs that impact bone remodeling will influence a bone's resistance to fracture.

In thinking about the determinants of bone strength and how bone strength changes with age, one must consider several important concepts. First, unlike most engineering materials, the bone is continually adapting to changes in its mechanical and hormonal environment, and is capable of self-renewal and repair. Thus, in

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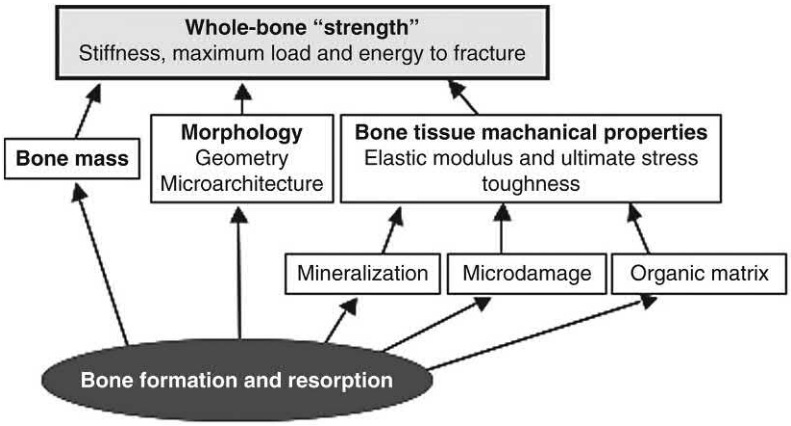

FIGURE 12.1 Determinants of whole-bone strength. Bone mass, morphology, and tissue-level material properties contribute to whole-bone strength. The balance between bone formation and resorption is the biologic process that mediates changes in bone mass, morphology, and matrix properties.

response to increased mechanical loading, a bone may adapt by altering its size, shape, and/or matrix properties. This type of adaptation is readily seen by the greater size of the bones in the dominant versus nondominant arm of tennis players[7] and of throwing athletes.[8] In addition, favorable changes in bone geometry may occur in response to deleterious changes in bone matrix properties.[9] Thus, the age-related changes in bone strength reflect continual skeletal response to altered biochemical and mechanical environments.

A second important concept concerns the hierarchical nature of the factors that influence whole-bone strength (Fig. 12.2). Alterations at the cellular, matrix, microarchitectural, and macroarchitectural levels may all impact bone mechanical properties, though not all to the same degree. In addition, the various factors are interrelated, and therefore changes in a single trait are unlikely to be the only predictor of changes in whole-bone mechanical behavior. Thus, the challenge is to identify (and measure) the factors that have the greatest impact on whole-bone strength and fracture risk.

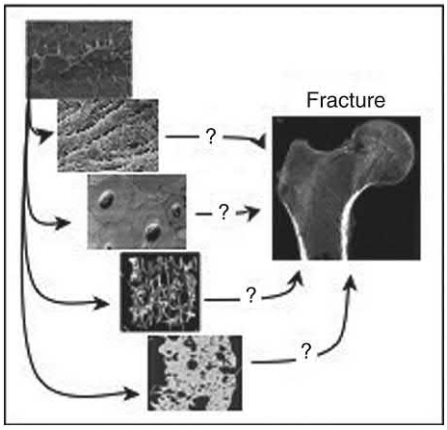

FIGURE 12.2 Hierarchical structure of bone. Changes in bone cellular activity influence the matrix, lamellar, and microarchitecture of bones. Alterations at all these different levels of structure may influence bone mechanical properties. Importantly though, the various factors are interrelated, and therefore a change at a single structural level is unlikely be the *only* predictor of changes in whole-bone mechanical behavior. The challenge is to identify and measure the factors that have the strongest association with whole-bone strength and fracture risk.

2.1 Structural Versus Material Properties of Bone

In discussing bone strength, it is important to distinguish between the material and structural properties of the bone. During any activity, a complex distribution of forces (or loads) is applied to the skeleton. With the imposition of these forces, bones undergo deformations. The relationship between the forces applied to the bone and the resulting deformations characterize the *structural behavior*, *or structural properties*, of the whole bone. Thus, the size and shape of the bone, as well as the properties of the bone tissue influence structural properties. In contrast to the structural behavior, the *material behavior*, *or material properties*, of bone tissue is independent of the specimen geometry. The material properties reflect the intrinsic biomechanical characteristics of cortical and trabecular bone. Although the biomechanical properties of the whole bone are functionally the most important outcome, assessing bone microarchitecture and tissue-level material properties may be helpful for understanding the mechanisms that underlie changes in whole-bone properties.

Importantly, in a heterogeneous material, such as bone, the definition of "material properties" is not altogether straightforward. In describing the material properties of bone, one could consider the mechanical properties of a single trabeculae, of the calcified bone matrix, or of small specimens of cortical or trabecular bone. For

purposes of this review, we consider bone "material" to include the calcified bone matrix, the marrow spaces in trabecular bone, and Haversian and Volkmann's canals in cortical bone. With this approach, we take a continuum mechanics view of bone, in that the specimen is small enough to be homogeneous (uniform), but large enough to include a sufficient number of trabeculae or osteons to characterize the overall material behavior.

2.2 Mechanical Properties of Bone Tissue Depend on Loading Direction

The elastic properties of *isotropic* materials, such as steel or rubber, are the same in all directions. The elastic properties of bone, however, depend on the orientation of the material with respect to the loading direction. Materials whose elastic properties are sensitive to loading direction are referred to as *anisotropic* materials. For example, cortical bone from the femoral diaphysis has a higher modulus and is stronger when loaded in the longitudinal direction than when loaded in the transverse direction.[10,11] The anisotropic nature of bone reflects its function as a load-bearing structure, as it is generally strongest in the primary loading direction. Hence, the degree of anisotropy in bone varies with anatomical site and functional loading.[12,13] For instance, trabecular bone from the vertebral body is highly anisotropic, being much stronger in the vertical direction than in the transverse direction,[14–16] yet trabecular bone from the iliac crest and central femoral head are nearly isotropic.[17,18]

2.3 Determinants of Biomechanical Properties of Trabecular and Cortical Bone

There is extensive literature describing the factors that influence the intrinsic (i.e., material) properties of trabecular and cortical bone, including the response not only to slow, monotonic loads, but also to high-rate impact loading, as well as to cyclic loading. The material properties of trabecular bone are influenced by many factors; however, the strongest determinants are apparent density (or volume fraction) and the microstructural arrangement of the trabecular network. Sampled over a wide range of densities, the stiffness and strength of trabecular bone are related to density in a nonlinear fashion, such that the change in strength is disproportionate (i.e., greater) than the change in density.[19–22] For example, a 25% decrease in density, approximately equivalent to 15 years of age-related bone loss, would be predicted to cause a 44% decrease in the stiffness and strength of trabecular bone. However, given the heterogeneous nature of trabecular bone, it is clear that density alone cannot explain all of the variation in trabecular bone mechanical properties. Both empirical observations and theoretical analyses indicate that trabecular microarchitecture contributes to bone mechanical properties. In particular, the degree of alignment of trabeculae with the axis of loading is a key contributor to bone mechanical behavior.[23,24]

The primary determinants of the biomechanical properties of cortical bone include porosity and the mineralization of the bone matrix. Indeed, over 80% of the variation in cortical bone stiffness and strength is explained by a power law relationship with mineralization and porosity as explanatory variables.[25–28] Some studies show that with increasing age, the degree of mineralization of the matrix increases, leading to stiffer, but more brittle material behavior,[29,30] although other studies indicate no age-related changes in the degree of mineralization.[31] McCalden et al. report that cortical porosity alone explains over 75% of the variability in the ultimate stress of cortical bone.[28] Other properties that influence cortical bone mechanical behavior include, but are not limited to, its histologic structure (primary, lamellar vs. osteonal bone), the collagen content and orientation of collagen fibers, extent and nature of collagen crosslinking, the number and composition of cement lines, and the presence of fatigue-induced microdamage.[32–37]

3 CONTRIBUTION OF BONE GEOMETRY TO BONE STRENGTH

With regard to whole-bone biomechanical behavior, the overall size of the bone (i.e., mass), as well as its morphology (i.e., ratio and distribution of cortical and trabecular bone mass) play important roles. Consistently, laboratory testing of the strength of human cadaveric vertebra, distal radii, and proximal femora has shown, not surprisingly, that larger bones are stronger than smaller bones.[38–42]

The loads applied to the skeleton generally are a combination of compression or tension forces with bending or torsional moments. The resistance to bending and torsional loading is particularly important, as the highest stresses in the appendicular skeletal are due to these loading modes. The most efficient design for resisting bending and torsional loads involves distributing the bone material far from the neutral axis of bending or torsion (generally this neutral axis is near the center of the bone). The distribution of mass about the neutral bending axis is quantitatively described by a geometric property termed the *area moment of inertia*. Importantly, the area moment of inertia of a solid circular bar is proportional to its diameter to the fourth power (i.e., moment of inertia α diameter).[4] Thus, small increases in the external diameter of a long bone can markedly improve its resistance to bending and torsional loading. The structure of the femoral neck is a good example demonstrating that the geometry of a bone reflects the forces that are applied to it. For example, the region of the femoral neck

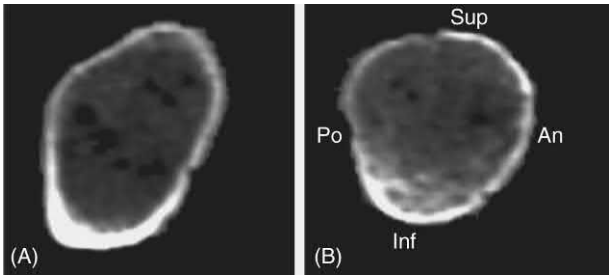

FIGURE 12.3 Cross-sectional computed tomography (CT) images of the femoral neck of a cadaveric hip. (A) Cross-section of the femoral neck adjacent to the shaft is elliptical, with the longer diameter in the superior-inferior (*Sup–Inf*) direction and with thicke cortical bone inferiorly to minimize bending stresses. (B) Cross-section of the femoral neck near the femoral head, showing a more circular shape [more area in the posterior-anterior direction (*Po–An*) than (A)] and largely trabecular bone, with uniform cortical thickness around the perimeter to withstand compressive stresses.

adjacent to the shaft is elliptical, with the longer diameter in the superior–inferior direction and with thicker cortical bone inferiorly to minimize *bending* of the femoral neck during the stance phase of walking (Fig. 12.3). In comparison, near the femoral head, where the stresses are mainly compressive, the femoral neck cross-section is largely circular and comprises trabecular bone, with uniform cortical thickness around its perimeter (Fig. 12.3).

3.1 Age-Related Changes in Bone Size and Shape

It is interesting to consider how age-related changes in bone geometry in part preserve whole-bone strength. Considerable evidence indicates that age-related declines in the material properties of bone tissue are accompanied by a redistribution of cortical and trabecular bone. Specifically, in the appendicular skeleton these changes involve endosteal and intracortical resorption along with periosteal apposition, leading to an age-related increase in the diameter of long bones but a decrease in cortical thickness.[43–45] This increase in the outer diameter helps to maintain the resistance to bending and torsional loads. It is generally thought that men undergo this pattern of favorable geometric adaptation mentioned previously to a greater extent than women, and that this may contribute to lower fracture rates in elderly men than women[43,44,46–50]; however, data employing three-dimensional (3D) quantitative computed tomography (QCT) challenge this paradigm.[51,52] A cross-sectional study by Riggs et al.[51] examined 373 women and 323 men aged 20–97 years and showed that whereas women have greater declines in volumetric bone density, both men and women show similar age-related increases in bone area and moments of inertia at the femoral neck and vertebral body, demonstrating that the extent to which bone geometry exhibits favorable geometric changes with aging is similar in men and women. A similar finding was reported in a cross-sectional study in men and women from Iceland, where femoral neck cross-sectional area increased by about 2% per decade in both sexes.[52] In contrast, longitudinal studies using QCT have been unable to detect changes in femoral neck dimensions.[53,54] To be able to detect such small increases in bone geometry with advancing age likely requires long-duration follow-up. For example, prospective studies of forearm geometry in women at menopause and with regular measurements up to 28 years afterward report that periosteal expansion is present in the first postmenopausal decade but not at later ages.[55,56] Moreover, a longitudinal study using single-energy X-ray absorptiometry measurements of the forearm in postmenopausal women over approximately 7 years found that, although periosteal expansion occurs, it does not compensate fully for endosteal resorption, leading to an age-related decline in estimated bone strength.[57] It is clear that carefully conducted longitudinal studies over several years duration, including both men and women, are needed to sort out the ability of different skeletal sites to increase their size to compensate for declines in bone density.

In sum, the sex-specific nature of age-related changes in bone geometry, particularly periosteal expansion, remain controversial. The discrepancies in findings related to sex-specific age-related changes in bone geometry may be attributed to several factors. Most importantly, many of these studies use a cross-sectional design, thereby possibly introducing secular changes that confound the data and eliminate the possibility of demonstrating a causal relationship with age. In addition, differences in methodology (direct vs. in vivo measurements), subject populations (archaeological vs. modern human specimens vs. in vivo studies), and measurement site (femur vs. radius vs. tibia) likely contribute to the conflicting findings.

4 AGE-RELATED CHANGES IN TRABECULAR AND CORTICAL BONE MICROARCHITECTURE

With aging, the coupled process of bone remodeling where bone formation equals resorption becomes unbalanced, with bone resorption exceeding formation. This imbalance in remodeling leads to a decline in bone mass overall. In particular, trabecular bone density declines profoundly with increasing age: by 45%–56% in the lumbar spine and femoral neck from age 20–90.[51] Perhaps more important than the decline in bone mass is the deterioration of trabecular and cortical microstructure that results from this net imbalance in remodeling.

In trabecular bone, the imbalance in bone remodeling leads to decreased trabecular thickness, trabecular perforation, and loss of individual trabecular elements. In

particular, whereas trabeculae aligned with the primary loading direction are maintained longer, those that are not aligned with the primary loading direction are preferentially resorbed, leading to greater structural anisotropy with increasing age. This phenomenon is especially prominent in the vertebrae, where horizontally oriented trabeculae are resorbed first, leaving behind vertically oriented trabecular struts that are unsupported horizontally and thus susceptible to failure by buckling[58–60] (Fig. 12.4). In addition, more bone resorption cavities weaken trabecular architecture, leading to declines in bone strength beyond what would be predicted simply by the decline in bone volume.[61,62]

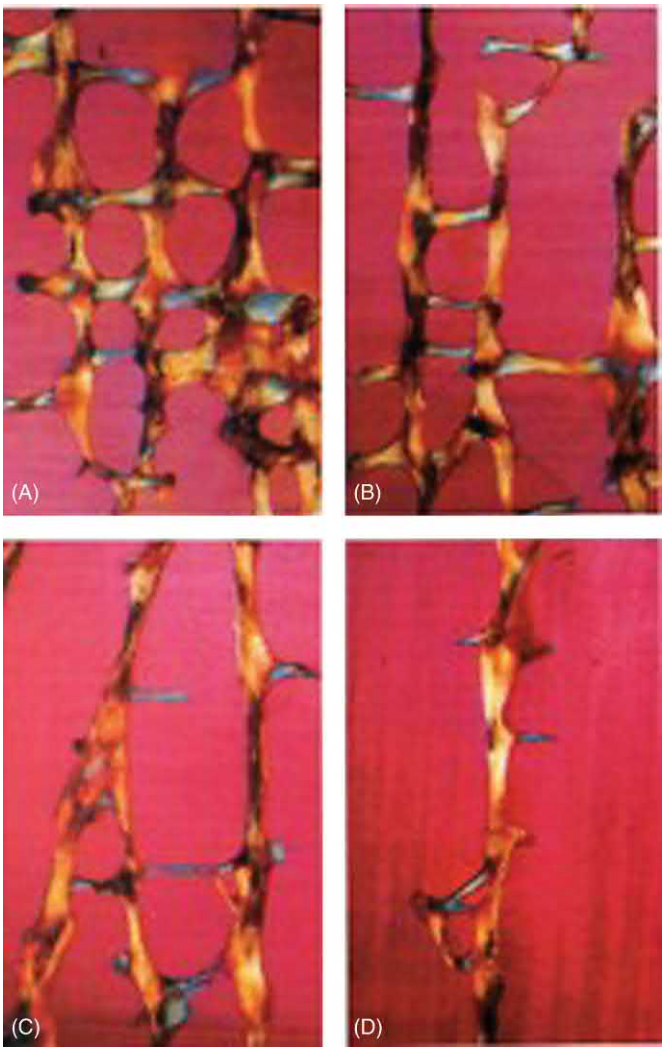

FIGURE 12.4 Age-related changes in vertebral trabecular architecture. Polarized light image of human vertebral trabecular bone in (A) 50-year-old man, (B) 58-year-old man, (C) 76-year-old man, and (D) 87-year-old woman. Note the progressive loss of horizontally oriented trabeculae, leading to a wider separation of trabecular elements, and unsupported vertically oriented trabeculae. Original magnification 8×. *Source: From Mosekilde L. Age-related changes in vertebral trabecular bone architecture—assessed by a new method.* Bone *1988;***9***:247–50, with permission.*[58]

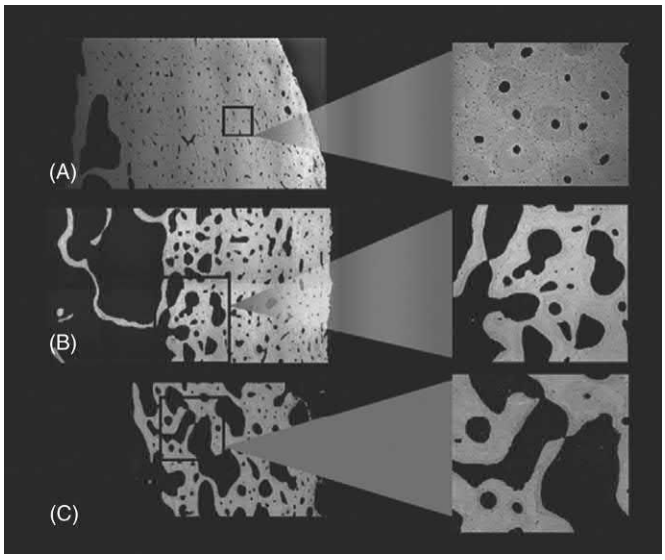

FIGURE 12.5 Age-related changes in femoral cortical structure. Micrograph of femoral subtrochanteric region in women aged (A) 29 years, (B) 67 years, and (C) 90 years. Note the small, regularly shaped pores in the young woman compared to large, irregular pores located toward the endocortical surface, leaving "trabecularized" remnants of the former cortical bone. *Source: From Zebaze RM, Ghasem-Zadeh A, Bohte A, et al. Intracortical remodeling and porosity in the distal radius and post-mortem femurs of women: a cross-sectional study.* Lancet *2010;***375***(9727):1729–36, with permission.*[66]

In cortical bone, the remodeling imbalance favoring resorption leads to a marked age-related increase in cortical porosity, particularly on the inner third of the cortex of long bones and in the femoral neck.[28,63–65] Intracortical remodeling near the endocortical surface appears to result in a "trabecularization" of the cortex as cortical pores coalesce, leaving remnants of cortical bone that resemble trabeculae[66] (Fig. 12.5). Both endocortical resorption and this trabecularization of the cortex lead to thinning of the cortex.

Trabecular bone loss begins early in life[67] and may slow with increasing age, as there are fewer and fewer trabecular surfaces for resorption to take place, and fewer remaining trabeculae to be resorbed. In contrast, cortical bone loss may begin later in life, but the process of intracortical resorption actually creates more surfaces for resorption, thereby allowing further resorptive activity. The consequence of this pattern is that much of the bone loss later in life is cortical bone.[66] Importantly, reflecting the importance of loading on the skeletal, the patterns of bone loss are not uniform neither between skeletal sites nor even within a given bone. For example, representative proximal femur images from two women, a 40-year old and a 77-year old (Fig. 12.6), show that at by age 40 there has clearly been trabecular bone loss in the trochanter and superiorly in the femoral neck, but the cortical bone is still preserved. In comparison, in the 77-year old there has been profound trabecular bone loss

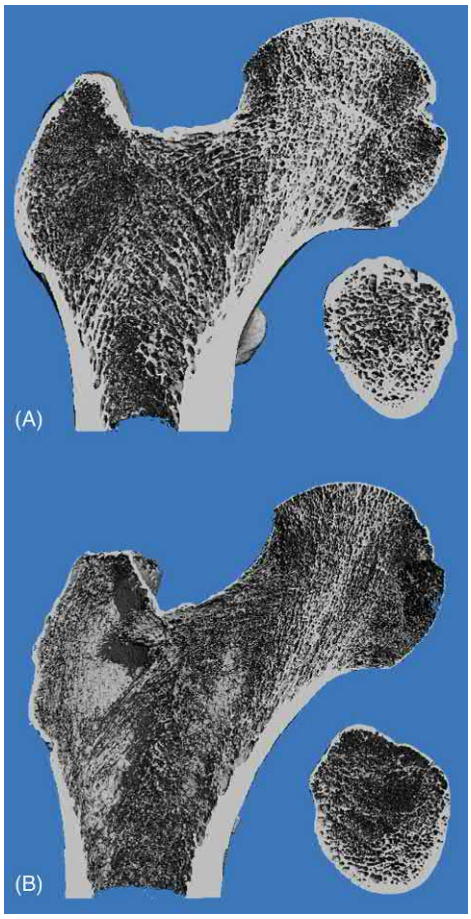

FIGURE 12.6 Age-related changes in trabecular and cortical bone structure in human proximal femur. These 3D models of cadaveric hips were made using high-resolution micro-CT. Midcoronal cross-section and cross-section through femoral neck of (A) a 40-year-old woman and (B) a 77-year-old woman, respectively. Note the extensive trabecular and cortical bone changes with increasing age.

and distinct cortical bone loss. However, in the weight-bearing inferior femoral neck both trabecular and cortical bone are preserved.

Longitudinal studies using in vivo high-resolution peripheral quantitative computed tomography (HR-pQCT) imaging of the distal radius and distal tibia have confirmed that trabecular bone loss begins in young adults and continues throughout life, whereas cortical bone loss begins later in life.[67,68] Further, rates of bone architecture decline were greater in women than in men.[68] These in vivo studies provide insight into the compartment-specific changes in bone microarchitecture that may contribute to fracture risk. To date, all studies have been conducted in Caucasian populations, and new data are needed to delineate longitudinal changes in bone microarchitecture in other race and ethnic groups, as several cross-sectional studies indicate that differences in bone microarchitecture may contribute to variation in fracture rates seen among different race/ethnic groups.[69–71]

5 CONTRIBUTION OF BONE MICROARCHITECTURE TO BONE STRENGTH

5.1 Trabecular Microarchitecture

Although bone volume fraction is among the strongest predictors of the mechanical behavior of trabecular bone, both empirical observations and theoretical analyses show that trabecular microarchitecture influences trabecular bone strength as well.[21,22,72] Trabecular architecture can be described by the shape of the basic structural elements and their orientation. The trabecular structure is generally characterized by the number of trabeculae in a given volume, their average thickness, the average distance between adjacent trabeculae, and the degree to which trabeculae are connected to each other. Previously, assessment of trabecular microarchitecture was possible only by 2D histomorphometry. However, newer high-resolution micro-CT and magnetic resonance imaging allow for 3D assessment of trabecular structure on excised bone specimens and in vivo.[5]

Laboratory studies have demonstrated moderate-to-strong correlations between trabecular bone architecture and biomechanical properties of trabecular bone.[12,73–76] Generally, however, trabecular bone microarchitecture is strongly correlated with trabecular bone volume[73,74,77] and therefore discerning the independent effects of specific architectural features on bone mechanical properties is challenging. Nonetheless, Ulrich et al. reported that including indices of trabecular architecture assessed by 3D micro-CT enhanced prediction of the biomechanical properties of human trabecular bone.[12] To further address this issue, analytical studies have been used to investigated how specific changes in trabecular architecture may influence trabecular bone mechanical behavior.[78–80] For example, Silva and Gibson employed an analytical model of vertebral trabecular bone, and reported that for the same decline in bone mass, loss of trabecular elements was 2–5 times more deleterious to trabecular bone strength than thinning of the trabecular struts, implying that maintaining connectivity of the trabecular network is critical.[78]

5.2 Age-Related Changes in Cortical and Trabecular Bone Material Properties

The age-related changes in cortical and trabecular bone microstructure lead to significant declines in elastic moduli and ultimate strength of cortical[28,29,81–84] and cancellous bone[15,60,85–88] in both men and women. In human cortical bone from the femoral middiaphysis, the tensile and compressive strengths and elastic moduli decrease approximately 2% per decade after age 20.[81] In addition, the incurred deformation and energy absorbed before fracture decrease approximately 5%–12% per decade,

suggesting the bone becomes more brittle and less tough with increasing age.[28,81,84] Moreover, the energy required to fracture a cortical bone specimen under *impact* loading decreases threefold between the ages of 3 and 90.[89]

Human cancellous bone exhibits an even more profound age-related decline in material properties.[15,85–88] For example, the elastic modulus, ultimate strength, and toughness of vertebral trabecular bone decline approximately 12%–15% per decade.[86] Aging is characterized by a marked decline in trabecular bone volume at all skeletal sites. This loss of trabecular bone volume is likely responsible for much of the decline in trabecular material properties, as there is a nonlinear relationship between bone density and strength,[19–21,86,90,91] such that a given change in bone density leads to relatively greater change in trabecular bone biomechanical properties. For example, the vertebral trabecular bone volume declines approximately 50% from ages 20–80, while vertebral trabecular biomechanical properties (compressive elastic modulus, ultimate stress, and energy to failure) decrease by approximately 75%–90%.[86]

5.3 Contribution of Bone Structure to Vertebral Strength

The vertebral body is characterized by a central core of cancellous bone surrounded by a thin covering of condensed trabecular bone (often referred to as a *cortical shell*). In the spine, compressive and bending loads are transferred from the intervertebral discs to adjacent vertebral bodies. Therefore, age-related changes in the properties of the intervertebral disc, the vertebral centrum, and the vertebral shell can each influence the load-bearing capacity of the vertebrae. In addition to a profound decline in trabecular bone volume, the thickness of the outer shell decreases from approximately 400–500 μm (age 20–40), to 200–300 μm (age 70–80), and to 120–150 μm in osteoporotic individuals.[92] This change in vertebral morphology likely influences the way that loads are transmitted throughout the spine. For instance, whereas the relative contributions of the vertebral centrum and shell to overall vertebral strength remain controversial, it is suggested that the vertebral cortical shell may account for 10%–30% of vertebral strength in healthy individuals, and due to decreased bone mass in the trabecular centrum, from 50%–90% in osteoporotic persons.[92–95] Yet, high-resolution finite element analyses indicate that the cortical shell has very little structural integrity when the vertebral trabecular centrum is absent, suggesting that the trabecular and cortical bone function together to determine vertebral strength.[96]

Several studies report that specific features of vertebral trabecular microarchtiecture contribute to whole-vertebral strength. Accordingly, addition of trabecular microarchitecture to bone mass improves the predicted vertebral strength,[23,97,98] and the bone volume fraction of *vertically oriented* trabeculae explained substantially more of the variation of whole-vertebral strength than does the total trabecular bone volume fraction (r^2 = 0.83 vs. r^2 = 0.59).[24] Furthermore, local variations in microstructure have also been associated with failure patterns in the vertebra, and accounting for the heterogeneous distribution of bone tissue throughout the centrum improves the prediction of strength compared to that based on mean density alone (r^2 = 0.75 vs. r^2 = 0.61).[99]

5.4 Contribution of Bone Structure to Proximal Femoral Strength

In addition to age,[100] loading rate,[101] and loading direction,[102–104] femoral geometry also influences the load-bearing capacity of the proximal femur. The relationship between femoral geometry and load-bearing capacity is not unexpected. As the load-bearing capacity is a *structural* property, it is influenced by the size of the specimen. Therefore, larger femurs have a greater load-bearing capacity. In fact, femoral neck area, neck width, and neck axis length are all positively correlated (r^2 = 0.21–0.79) with femoral failure loads.[100,102,105,106] The positive correlation between femoral neck length and femoral strength appears to contradict findings from clinical studies, where a longer hip axis length is associated with a greater risk of hip fracture.[107]

Cortical and trabecular bone properties both contribute to mechanical behavior of the human proximal femur. Evidence for an important role of trabecular bone in femoral strength is provided by a study of the microstructural failure mechanisms of the human proximal femur during a sideways fall impact, which showed that tissue-level failure starts in the trabecular bone.[108] Also, indices of trabecular architecture contribute independently of bone density to the prediction of femoral strength in vitro.[109] High-resolution finite element modeling of the elderly human femoral neck found that, while cortical bone supported up to 90% of the overall load in stance, it supported only about 60% of the load in a sideways fall, indicating that cortical and trabecular bone work in synergy to provide strength of the femur.[110] Additional studies are required to understand the complex relationship between hip geometry, the distribution of cortical and trabecular bone, and fracture risk.

6 CONTRIBUTION OF BONE STRUCTURE TO FRACTURE RISK IN HUMANS

Clinical studies support the importance of both bone size and microarchitecture as predictors of fracture risk. For example, lower cross-sectional area of the radius is

a risk factor for wrist fracture among both young girls[111] and postmenopausal women.[112] In addition, individuals with smaller vertebral bodies have an increased risk of vertebral fracture,[113–115] and a smaller femoral neck cross-section and lower cortical bone volume increase the risk of hip fracture in men even after adjusting for femoral neck BMD.[116]

The importance of bone microarchitecture has also been supported by clinical studies showing altered trabecular and cortical microarchitecture in subjects with fragility fractures compared to age-matched controls with no fractures.[117–120] For example, Ciarelli et al.[117] compared trabecular bone specimens from the femoral head of individuals who had suffered a hip fracture to age-matched cadaveric controls and showed that, after controlling for bone volume, trabecular bone from individuals who suffered hip fracture was more oriented in a single direction than bone from unfractured individuals. One interpretation of this finding is that the femoral trabecular bone from individuals with hip fracture was able to withstand unusual loading conditions to a lesser degree, such as would be expected during a sideways fall. Other studies have also shown altered trabecular microarchitecture among those with vertebral fracture,[118,120] and that the extent of microarchitectural deterioration is related to vertebral fracture severity.[121]

Furthermore, several cross-sectional studies have shown that in vivo measurements of trabecular or cortical bone microarchitecture at the distal radius and tibia are worse in women and men with a prior fragility fracture.[122–130] For example, Boutroy et al. were the first to demonstrate that, although osteopenic women with a history of fragility fracture had similar hip and spine BMD as other women with no prior fracture, they had worse trabecular bone architecture than those without prior fractures.[122] Similarly, premenopausal women with a recent distal radius fracture had worse trabecular bone microarchitecture than controls with no fracture, even after adjusting for forearm BMD.[131] A recent multicenter study including nearly 1400 postmenopausal women found that deficits in trabecular volumetric BMD and microarchitecture were associated with a history of fragility fracture, independent of hip BMD *t*-score.[130] Cortical porosity in the appendicular skeleton may also contribute to fracture risk, and in particular may be helpful in distinguishing women, who are at higher risk for fracture, with osteopenia by dual-energy X-ray absorptiometry areal bone mineral density (DXA-aBMD) measurements.[132,133] Cortical bone deficits at the proximal femur are also important, as specific changes in femoral neck cortical thickness, particularly a decline in thickness of the superoanterior region, are associated with hip fracture more strongly than BMD alone.[134] Furthermore, a study applying voxel-based morphometry to QCT images of the proximal femur found that compared to nonfractured controls, postmenopausal women who fractured their hips had relative deficits along the endocortical margin at both the superior and inferior aspects of the femoral neck, as well as in the intertrochanteric region.[135] Interestingly, the regions at the hip that are fracture related are similar to where age-related declines are most prominent as well, except the inferior femoral neck, which is largely preserved in normally aging women.[54]

7 CONCLUSIONS

Although fractures are associated with low BMD, many individuals who fracture do not have BMD in the osteoporotic range.[136,137] These observations indicate that a better understanding of the bone and nonbone factors that influence fracture risk may improve identification of those at the highest risk for fracture. This chapter outlined the key determinants of bone strength, focusing on the key role of bone morphology and microarchitecture. While it is clear that bone morphology and microarchitecture contribute to bone strength and fracture risk independently of BMD, it is only relatively recently that bone microstructure could be measured noninvasively. While initial cross-sectional studies support an important role for trabecular and cortical bone architecture in skeletal fragility, additional prospective studies are needed to clarify the clinical utility of bone structure measurements in vivo. There is marked heterogeneity in bone structure, even at a single skeletal site, and research is needed to determine the underlying contributors to this wide range in bone size, shape, and structure. In particular, there are few studies examining the genetic determinants of bone structure[138]; although this is likely to be an area of great interest with the growing availability of noninvasive imaging modalities to assess bone structure.

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CHAPTER

13

Overview of Joint and Cartilage Biology

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1 INTRODUCTION

The joints are specialized structures that connect the bones of the skeleton and support movement while at the same time restricting the range of motion between its different elements. The joints in the body are structurally and functionally divided into three different groups. The *synarthroses* or fibrous joints, such as the sutures between the bones of skull, allow very little or no movement. *Amphiarthroses*, typically found in the spine, have only limited mobility and are characterized by a fibrocartilaginous cap between the articulating bones. From the perspective of mobility, *diarthroses* or synovial joints are most important (Fig. 13.1). In these specialized organ-like structures, the bony bearings are capped with a thin layer of hyaline or articular cartilage while the joint cavity is lined by the *synovium* or synovial membrane. The joint capsule further encloses the joints. Intra- and extraarticular ligaments provide additional stability. Together, these different tissues allow smooth movement with very low friction and sufficient lubrication between joint surfaces while preventing dislocation. The shape of the synovial joints varies greatly from site to site. For instance, the glenohumeral joint in the shoulder and the acetabulofemoral joint in the hip typically have a ball-in-socket shape that allows rotation movements. Condyloid joints have a concave side articulating with a convex surface allowing flexion, extension, adduction, and abduction, for example, in the tibiofemoral joints of the knee and the radiocarpal joint of the wrists. Small joints in the hands and feet typically have a saddle form and allow similar directions of movement as the condyloid joints.

In this chapter, we discuss the development, anatomy, and function of the synovial joints; the main diseases affecting the joints; and give some insights into the rapidly developing aspects of joint-repair strategies.

2 JOINT DEVELOPMENT

Great progress has been made in understanding the basics of skeletal and joint development. Most data available provide insights into the developmental processes of the different skeletal elements and the molecular aspects of chondrogenesis and osteogenesis.[1,2] Less is currently known about the factors that specifically control joint development. This knowledge is particularly relevant as it provides a basis to better understand the molecular background of common joint disorders and diseases, as well as genetic syndromes associated with joint malformations.[3,4] Insights into mechanisms of joint development are also important for regenerative medicine and tissue engineering, which try to mimic nature's own mechanisms to restore a damaged tissue, a concept described as a biomimetic or developmental engineering approach.[5]

Joint development consists of different phases and events which all contribute to the future functional synovial joint.[1,6–9] These include patterning and joint specification, cell commitment, cell differentiation, joint cavitation, and integration of the joint with the rest of the skeletal elements. Part of joint development in particular the formation of the joint surface, the articular cartilage, and subchondral bone, is a postnatal and not a fetal event (Table 13.1).

2.1 Joint Development as a Part of Skeletal Development

To understand the specifics of synovial joint formation, these processes cannot be uncoupled from the general plan for the outline of the skeleton in vertebrate organisms.[1,2]

The primary skeleton is almost entirely cartilaginous and most of the cartilaginous template will be progressively replaced by bone during development

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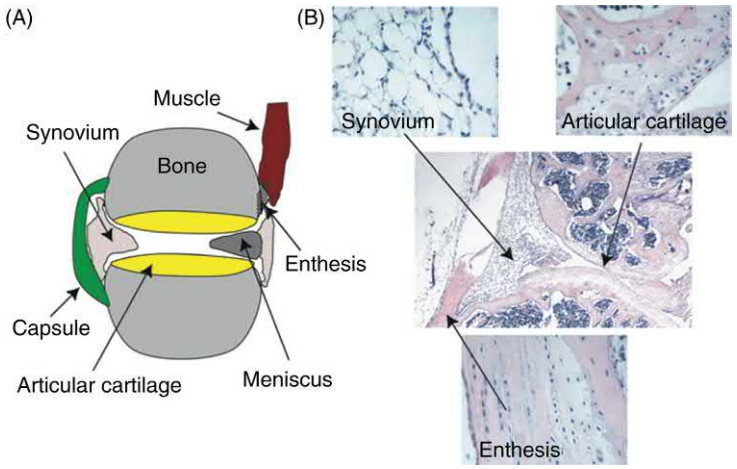

FIGURE 13.1 The anatomy of the synovial joint. (A) Schematic drawing of a synovial joint. The articular surfaces are connected by the synovium that consists of a thin pseudoepitheloid lining layer and a loose connective tissue sublining zone. In some joints a fibrocartilagenous meniscus is found. The joint is encapsulated. The enthesis is an anatomic region referring to the insertion of tendons and ligaments in the underlying bone. (B) Microscopic image of a knee joint in the mouse. Images of the synovium, the articular cartilage and the enthesis are further enlarged. *Source: Lories RJU, Luyten FP. Bone morphogenetic protein signaling in joint homeostasis and disease.* Cytokine Growth Factor Rev *2005;***16***(3):287–98.*[18]

and growth. This process is called endochondral bone formation (Fig. 13.2). Joints and additional bones will be formed concomitantly during fetal life and postnatal growth. The skeletal elements and the joints are derived from cell populations denominated as osteochondroprogenitor cells. However, as these cells also contribute to the development of other tissues, such as the *synovium*, the ligaments, and tendons; it was proposed to call them skeletogenic cells.[1]

During skeletogenesis, two different processes play an essential role: skeletal patterning which defines the position of the different skeletal elements and tissue specification. The skeletogenic cells are mainly derived from the embryonic mesoderm (lateral plate for the appendicular skeleton and paraxial mesoderm for the axial skeleton) although ectodermal neural crest cells contribute to the development of the cranial bones (Fig. 13.3). Patterning genes, such as the *Hox* (Homeobox) cluster define the position of the different skeletal elements[10,11] and master switch transcription factors, such as *Sox9* (SRY-box containing gene 9) and *Runx2* (runt related transcription factor 2) steer chondrogenesis and osteogenesis, respectively.[2,12] *Sox9* acts in a complex with functionally redundant *Sox5* and *Sox6*, whereas *Osx* (Osterix) is an essential transcription factor in osteogenic differentiation.[13]

Bone and cartilage are different tissues with distinct properties and function. Cartilage has a collagen type II, aggrecan, and other proteoglycan-rich extracellular matrix (ECM) giving it resilient properties.[14] In bone, type I collagen is prevalent and the matrix is mineralized, a process to which macromolecules, such as osteocalcin and bone sialoprotein contribute.[15] The skeletal elements form through well-defined branching of cell condensations (anlagen) in which chondrogenesis starts (Fig. 13.2). Growth of the differentiating anlagen occurs through shaping of the growth plate in the small diaphysis and later on in the epiphyses. The central diaphysis contains the primary ossification center whereas the epiphyses become the secondary ossification centers. During endochondral bone formation, progenitor or skeletogenic cells differentiate into chondrocytes and go through different phases of maturation to hypertrophy, finally becoming terminally differentiated cells undergoing apoptosis and being replaced by a calcified matrix and bone. As outlined later, the development of the articular cartilage does not follow this path and many aspects of its development and functional integration with the other skeletal tissues remains unknown.

2.2 Joint Site Determination

The first apparent sign of joint specification is the demarcation of the joint interzone, a region with more compact and tightly associated cells that forms between the branching cartilaginous elements[1,6,9] (Fig. 13.4). Gap junctions, specialized intercellular communication pores that allow the transport of inorganic ions and small hydrophilic molecules directly from cell to cell, connect the interzone cells. It is not clear whether the anlagen first develops as a continuous element in which the joint interzones are later defined or whether these zones are prespecified and never enter the sequence of skeletal element formation. Data looking at specific isoforms of the type II collagen gene transcript suggest that cartilage-associated collagen type IIB is never expressed in the joint interzone supporting the concept that the skeletal elements appear as entities different from the future joints.[8] The specification of the joint interzones may be

TABLE 13.1 Genetic Disorders in Mice and Men Linked to Genes Involved in Joint Development

Genes of interest	Mutations in mouse	Human genetic disorders	OMIM accession no. (www.omim.org)
Wnt4	Homozygous mutants exhibit impaired development of the kidney, pituitary gland, and female reproductive system. Mutants die within 24 h of birth.	Mullerian aplasia and hyperandrogenism Serkal syndrome	603490
Wnt9a	Neonatal lethality, altered chondrocyte maturation, cranial defects, and skeletal abnormalities including shortened appendicular long bones, partial joint fusions of carpal and tarsal elements, and chondroid metaplasia in synovial and fibrous joints.	None identified	602863
Wnt16	No data available.	None identified	606267
β-Catenin	Homozygous null embryos show anterior-posterior axis formation anomalies, but develop to E7. Multiple conditional mutations have shown defects in distinct stem cell types that result in proliferation defects, such as intestinal polyps, brain, and spinal cord size anomalies, etc.	Colorectal cancer Hepatoblastoma Hepatocellular carcinoma Ovarian cancer Pilomatricoma	116806
Gdf5	Homozygotes for null mutations exhibit slightly shortened long bones of the limbs, and drastically shortened bones of the feet, with some complete or partial fusions.	Acromesomelic dysplasia Hunter–Thompson type Brachydactyly, type C Chrondrodysplasia, Grebe type Fibular hypoplasia and complex brachydactyly Symphalangism, proximal Multiple synostoses syndrome 2 Osteoarthritis	601146
Gdf6	Homozygous null mice show multiple joint and skeletal patterning defects affecting the extremities, inner ear, and skull.	Klippel–Feil syndrome 1, autosomal dominant Microphthalmia, isolated 4	601147
Noggin	Homozygotes for a targeted null mutation exhibit failed closure of neural tube, exencephaly, wide club-shaped limbs, loss of tail vertebrae, shortened body axis, abnormal cartilage condensations, and lethality at birth.	Brachydactyly, type B2 Multiple synostosis syndrome 1 Stapes ankylosis with broad thumb and toes Symphalangism, proximal Tarsal–carpal coalition syndrome	602991
Chordin	Homozygotes for a targeted null mutation show some death prior to embryonic day 8.5, but most die perinatally with abnormalities of the skull, malformations of cervical, and thoracic vertebrae, cardiovascular defects, and absence of parathyroid and thymus.	None identified	603475
Sox5	Homozygous null mice fail to breathe and die at birth exhibiting a narrow thoracic cage, irregularly mineralized sternum, cleft secondary palate, and delayed bone mineralization.	None identified	604975
Sox6	Homozygotes for null mutations exhibit cardioskeletal myopathy, cardiac blockage, delayed growth, and early postnatal lethality.	None identified	607257
Sox9	Null mutant heterozygotes and conditional knockout mutants display perinatal lethality with cleft palate, hypoplasia, and distortion of numerous cartilage-derived skeletal structures, and premature mineralization in many bones. Specific conditional knockout mutations are sex reversed.	Acampomelic campomelic dysplasia Campomelic dysplasia Campomelic dysplasia with abnormal sex reversal	608160

determined by the expression of a number of genes that are associated with skeletal patterning, such as the *Hox* gene cluster. For instance, *Hoxa* genes appear to determine skeletal elements along with the proximodistal axis whereas *Hoxd* genes define the place along with the anteroposterior axis in the limb.[10,11] Boundaries or gradients between the expression of the different Hox genes could determine the position of the joint interzones.[7] From the molecular perspective, joint interzone cells are characterized by the expression of specific markers, including different Wnts [*Wnt4*, *Wnt9a* (formerly *Wnt14*), and *Wnt16*],[9,16,17] bone morphogenetic proteins (BMPs), and

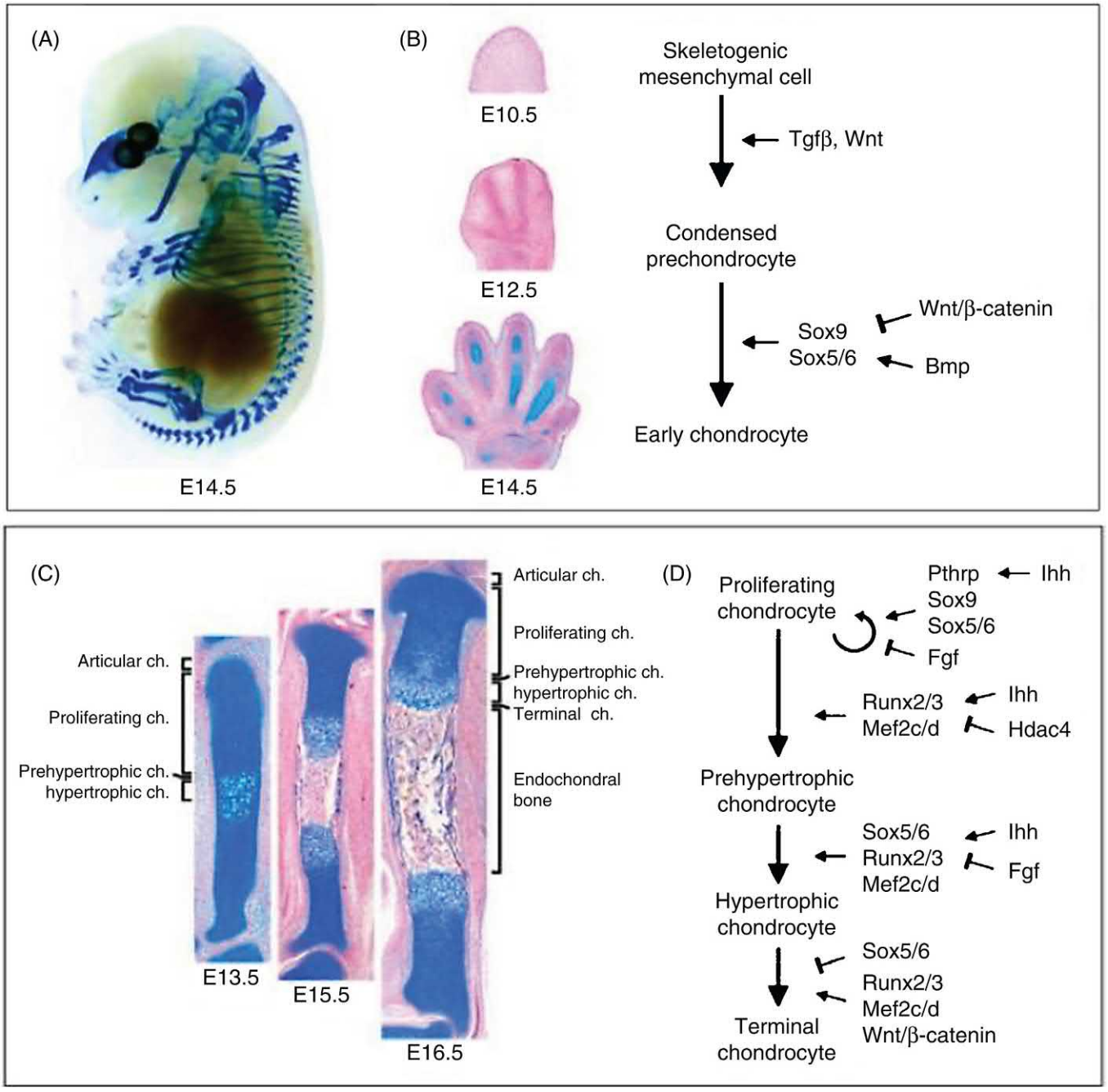

FIGURE 13.2 **Endochondral bone formation.** (A) Alcian blue staining of a mouse embryo at E14.5 demonstrates that chondrocyte differentiation of skeletogenic cells leads to the formation of a primary skeleton that is entirely cartilaginous. (B) Sections through the developing paws of mouse embryos illustrate the major steps of early chondrogenesis. At E10.5, the limb bud is filled with skeletogenic cells. By E12.5, some of these cells have formed precartilaginous condensations that prefigure the future digits. By E14.5, condensed prechondrocytes have undergone chondrocyte early differentiation. The sections are stained with alcian blue and nuclear fast red. (C) Sections through a mouse embryo tibia illustrate the development of growth plates and endochondral bone. At E13.5, early chondrocytes in the center of cartilage primordia undergo prehypertrophic and hypertrophic maturation. They reach terminal maturation and are replaced by endochondral bone by E15.5. Later on, growth plates maintain themselves and elongate developing bones. Chondrocytes keep proliferating and give rise, layer by layer, to maturing chondrocytes. These cells which eventually die and are replaced by bone. The sections are stained with alcian blue and nuclear fast red. (D) Schematic presentation of the molecular control of growth plate chondrocytes. *Source: Reproduced from Lefebvre V, Bhattaram P. Vertebrate skeletogenesis*. Curr Top Dev Biol *2010;***90***:291–317.*[1]

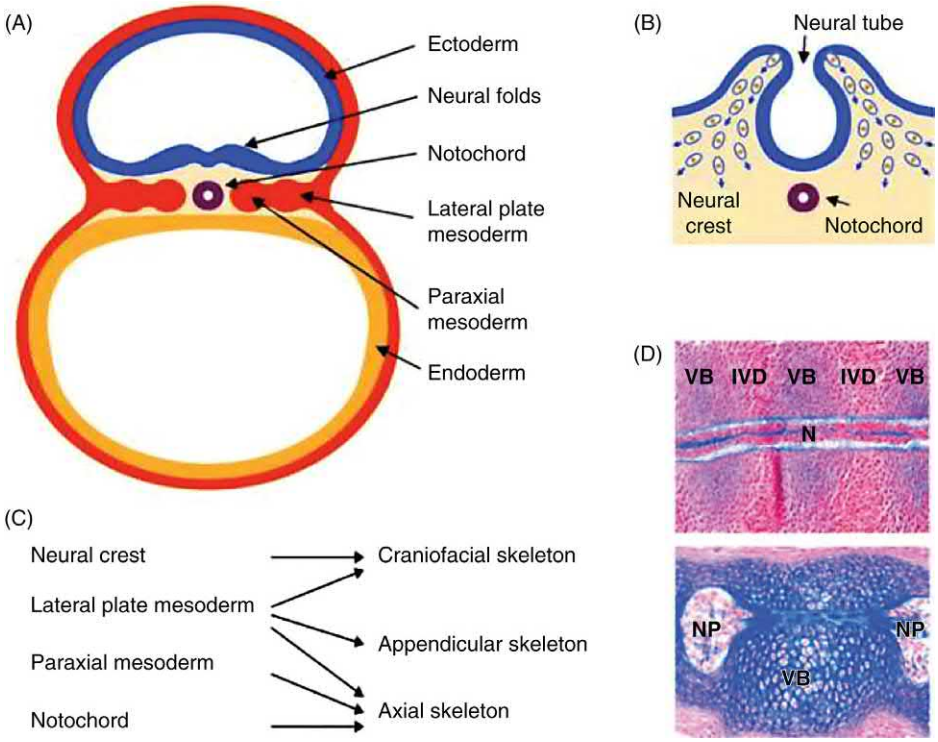

FIGURE 13.3 Origin of skeletal cells in the vertebrate embryo. (A) Schematic of a cross section through a mouse embryo soon after gastrulation at day 8 of development (equivalent to gestation day 17 in humans). The three germ layers are seen: ectoderm, endoderm, and mesoderm. Ectoderm-derived neural folds are rising. The mesoderm has formed the notochord and is starting to form lateral plate and paraxial derivatives on either sides of the midline. (B) Schematic showing the delamination of neural crest cells from the neural folds at the time of neural tube closure. These cells are starting to migrate inside the embryo [(*small arrows* (blue arrows in the web version)], where they will participate in the formation of various structures. (C) Schematic showing the contribution of the neural crest, lateral plate mesoderm, paraxial mesoderm, and notochord to the three major parts of the skeleton. (D) Mid-sagittal sections through the notochord of mouse embryos at the gestation days 12.5 (E12.5, top) and E15.5 (bottom). The E12.5 notochord is a rod-like structure that becomes surrounded by the mesenchymal cell condensations of the prospective vertebral bodies (VB) and intervertebral discs (IVD). E15.5 vertebral bodies are cartilaginous and notochord cells have migrated into the intervertebral disc spaces, where they have formed nuclei pulposi (NP). Sections are stained with nuclear fast red and with alcian blue, which is specific of the notochord and cartilage extracellular matrix (ECM). *Source: Lefebvre V, Bhattaram P. Vertebrate skeletogenesis.* Curr Top Dev Biol *2010;***90***:291–317.*[1]

related growth and differentiation factors (GDFs; *Gdf 5* and *6*), BMP antagonists (Noggin and Chordin),[6,18] the type II transforming growth factor beta receptor[19] *Sox5*, *6*, and *9*,[20] as well as type II collagen but not matrilin-1,[21] supporting differences between the stable articular chondrocytes and developing, transient chondrocytes in the growth plate.

2.3 What Happens in the Joint Interzone?

The joint interzone contains different populations of cells that contribute to the development of the synovial joints (Fig. 13.4). Articular progenitor cells are likely derived from the skeletogenic precursors but take a different path when present in the joint interzone.[6–8] The three-layered interzone consists of two chondrogenic layers and the densely packed cells in between where cavitation will occur.[22] Specific dissection of joint interzone cells has demonstrated the chondrogenic activities of the outer layers, which are in continuity in vivo with the epiphyseal ends with which they may integrate. The inner layer is more likely to be the source of the articular chondrocytes. Less is known about the source of the cells that will form the other tissues of the joint, such as the *synovium* and the ligaments.[8,23]

Gdf5 was one of the first genes associated with the joint interzone and its essential role in the development of the synovial joint is demonstrated by the severe skeletal phenotypes, including multiple joint fusions in mice and human, associated with loss-of-function mutations in this gene.[24–27] There is substantial functional overlap with redundancy between GDF5 and GDF6, and the phenotype of the double knockout mice is more severe than that of each single knockout model.[28] However, GDF5 is not the joint-inducing factor as in vitro and in vivo experiments have rather supported its chondrogenic properties.[3,29] Moreover, GDF5 is dynamically expressed during skeletal development, first in the developing anlagen where it appears to stimulate chondrogenesis and later in the joint interzones where it

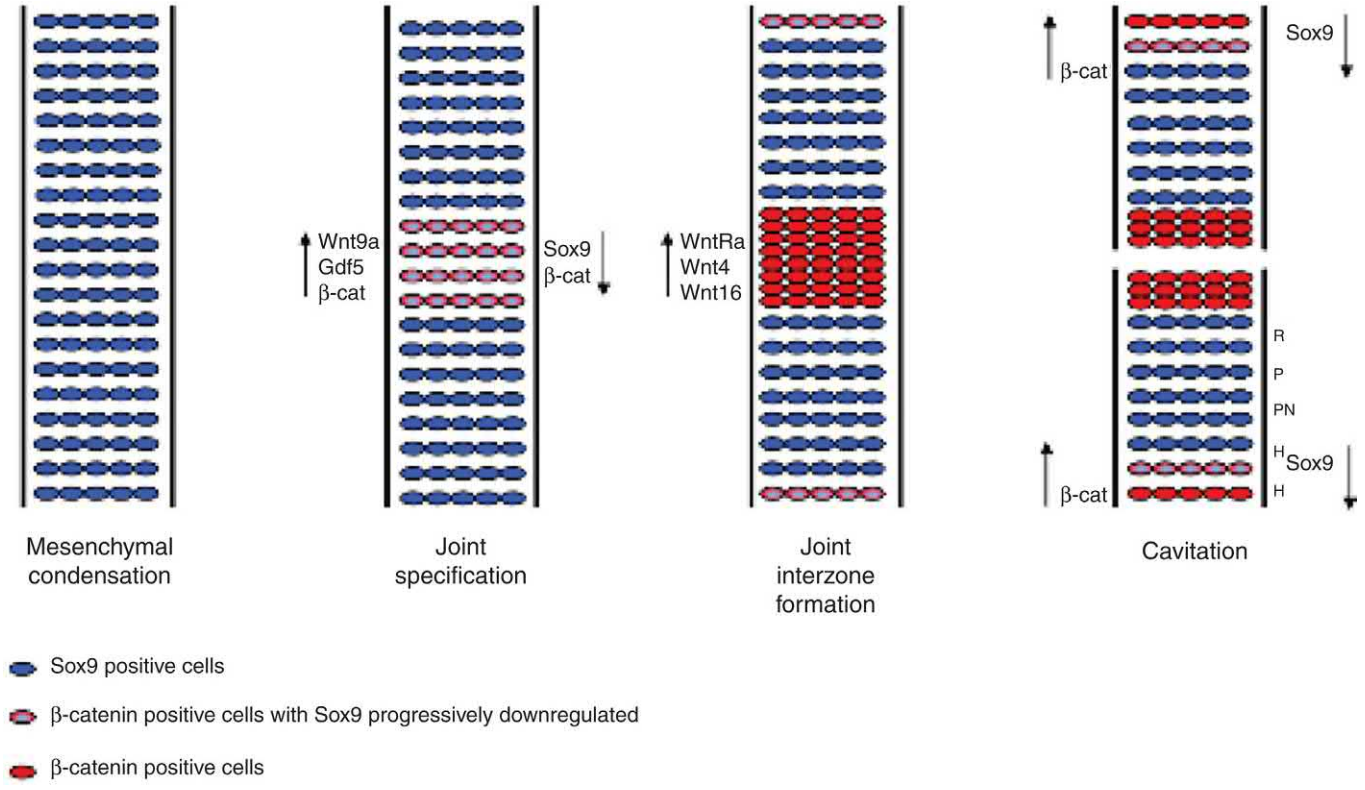

FIGURE 13.4 A model of joint formation. A mesenchymal condensation expresses *Sox9* [black (blue in the web version) ovals]. Upon *Wnt9a* signaling, transcription of β-catenin and *Gdf5* is induced while *Sox9* and collagen II repressed [*light gray* (red/blue in the web version) ovals]. Next, cells in the joint interzone cease expressing *Sox9* and maintain the expression of β-catenin [*gray* (red in the web version) *ovals*]. Finally, the cavitation process takes place with physical separation of the cartilaginous element. Concomitant with that process, *Sox9* is repressed and β-catenin induced in hypotrophic chondrocytes. *Arrows up*, induction; *arrows down*, repression; *black* (blue in the web version) *ovals*, *Sox9* positive cells; *light gray* (blue/red in web version) *ovals*, *Sox9* and β-catenin positive cells; *gray* (red in the web version) *ovals*, β-catenin positive cells. R, P, PH, and H indicate resting, proliferating, prehypotrophic, and hypotrophic chondrocytes, respectively, B—bone. *Source: Luyten FP, Tylzanowski P, Lories RJ. Wnt signaling and osteoarthritis.* Bone *2009;***44***(4):522–27.*[4]

may affect differentiation processes downstream of factors that initiates joint development. BMP antagonists and GDF5 processing enzymes may play a specific role in defining boundaries or gradients of BMP signaling. Of interest, the skeletal and joint consequences of loss of function in the noggin gene are far more severe than that of the chordin gene suggesting that these different BMP antagonists are not functionally redundant.[30,31]

Wnt9a is another gene that is specifically expressed within the joint interzone.[16,17] Viral overexpression in the developing chick limb induces local changes resembling the definition of a joint interzone. Moreover, *Wnt9a* is a factor that negatively affects chondrogenesis. Loss of *Wnt9a* in mice does not affect joint induction, but results in synovial chondroid metaplasia in some joints.[9,32] Other Wnts appear are also involved, in particular Wnt4 and Wnt16.[17] Conditional ablation of the canonical Wnt signaling master switch β-catenin leads to abnormal joints.[33] The current dataset therefore suggests that Wnts are required but are not sufficient for a full joint to develop.

Indian hedgehog (IHH) signaling is another pathway of interest as *Ihh* ablation leads to joint defects, mainly in the distal elements.[34] This suggests that IHH could be a critical mediator not only in the growth plate where it determines the speed of proliferation and differentiation,[1,2] but also in the communications between the joint interzone and the secondary ossification centers in the epiphyses. Another molecule of interest is α5β1 integrin.[35] Both gain- and loss-of-function approaches in developing limb models have suggested that blocking this integrin results in the formation of joint interzone-like structures whereas misexpression leads to joint fusions. However, these data still need to be corroborated in in vivo systems to fully define the extent of the role of these integrins.

2.4 Joint Cavitation

The process of cavitation that gives rise to the synovial space remains poorly understood. Cell death was thought for a long time as an essential factor but this hypothesis has not been convincingly demonstrated as

signs of cell death and apoptosis are only found in small and limited areas in the developing joints.[8,22,36,37] Current concepts therefore rather suggest that rearrangement of the ECM and production of hyaluronan are the essential factors in the cavitation process.[8] However, evidence from developmental models also provides support for the long-standing hypothesis that biomechanical factors (movement) are essential[38] whereas initial cell death in a time- and place-restricted manner may contribute to the specific initiation of events.[7] Picnotic dark cells previously associated with cell death and apoptosis are now considered to be precursors cells of the superficial layer of the articular cartilage.[22] The changes in hyaluron synthesis have been linked to activation of the mitogen activated protein kinases[39] and to upstream fibroblast-like growth factor signaling.[40,41]

2.5 The Differentiation of the Articular Cartilage and Other Joint Structures

The articular cartilage mainly develops postnatally and the chondrocytes that are found within this tissue have specific properties as compared to the developing chondrocytes in the anlagen and growth plates.[7] In contrast to the latter cells, articular chondrocytes do not undergo terminal differentiation toward hypertrophic cells. Despite articular cartilage being extremely important from a clinical and functional perspective, surprisingly little is known about the specific factors and processes that steer its formation. Current evidence suggests that chondrogenic transcription factors *Sox5* and *Sox6* are required.[20] *Sox9* likely also plays a role but it is not clear whether it is really essential.[1] Other molecules suggested to play a role include notch signaling[42] and the transcription factor ERG (v-ets erythroblastosis virus E26 oncogene homolog).[43] Further evidence suggests that the latter acts as a factor that maintains a pool of chondrogenic cells. Similarly, Wnt–β-catenin signaling may have a role in preventing chondrocyte differentiation and contribute to the differentiation of progenitors that will not become articular chondrocytes.[44] Some observations suggest that most of the cartilage present at birth in the epiphyses bordering the joint will be replaced by bone and that the articular cartilage itself is formed in the first 3 months of postnatal life.[45] The articular cartilage therefore appears to be formed by appositional growth. Of interest, both the superficial zone of adult cartilage and the synovium contain cell populations that fit the definition of mesenchymal stem cells (MSCs), which could play a role in cartilage formation.[46–48] More recently, in lineage tracing experiments, it was reported that in mice *Prg4*-expressing cells may include both multipotent articular stem cells and lubricin-producing cells.[49] These findings further contribute to our understanding of the articular joint surface development, and illustrate the need for further research to more fully dissect the lineage and functional relationships between joint surface cells and the underlying articular chondrocytes.

Even less is known about the factors contributing to the development of ligaments and *synovium*. Of interest, mice with a naturally occurring loss-of-function mutation in *Gdf5* [brachypodism (bp) mice] lack different ligaments supporting a role for GDF5 in their development.[24,50] The synovium is lined by the synovial fibroblasts, which specifically express cadherin-11.[51] Genetic deletion of cadherin-11 affects the anatomy of the synovium. In the absence of the adhesion molecule a clearly defined lining layer is not found.

2.6 Integration of the Joints with the Skeleton and Joint Shape Morphogenesis

Skeletal development clearly entails more than the definition and patterning of the different elements and subsequent specification of the skeletal tissues. Joint development and integration with the skeletal elements is essential to build the skeleton and to make it functional. In this concept both the primary, secondary ossification centers, and the developing joint are likely to act as signaling centers that define each other's boundaries. The interaction between the developing joint with the articular cartilage and the secondary ossification center that will become the subchondral bone is critically important. Within the secondary ossification center a zone of new bone is formed between the growth plate and the articular cartilage. MMP14 is a critical factor in establishing this zone[52] and interestingly it is also involved in the remodeling of the enthesis, the anatomical zone in which tendons and ligaments insert into the bone.[53] The boundary between the articular cartilage and the subchondral bone appears to be dependent on an IHH/parathormone related peptide loop which may confer the resistance of the articular chondrocytes toward hypertrophy.[54]

3 JOINT ANATOMY

The synovial joint is composed of different tissues that function together as a complex organ. Among these, the articular cartilage and the *synovium* are the structures that provide the potential to move. Other parts of the joints include ligaments, capsule, menisci, and tendons and all play a role in supporting or limiting movement.

3.1 The Articular Cartilage

The articular cartilage is a nonvascular connective tissue comprising the articular chondrocytes as a unique cell population that are producing a specific ECM in which they are embedded (Fig. 13.5). This ECM is essential for

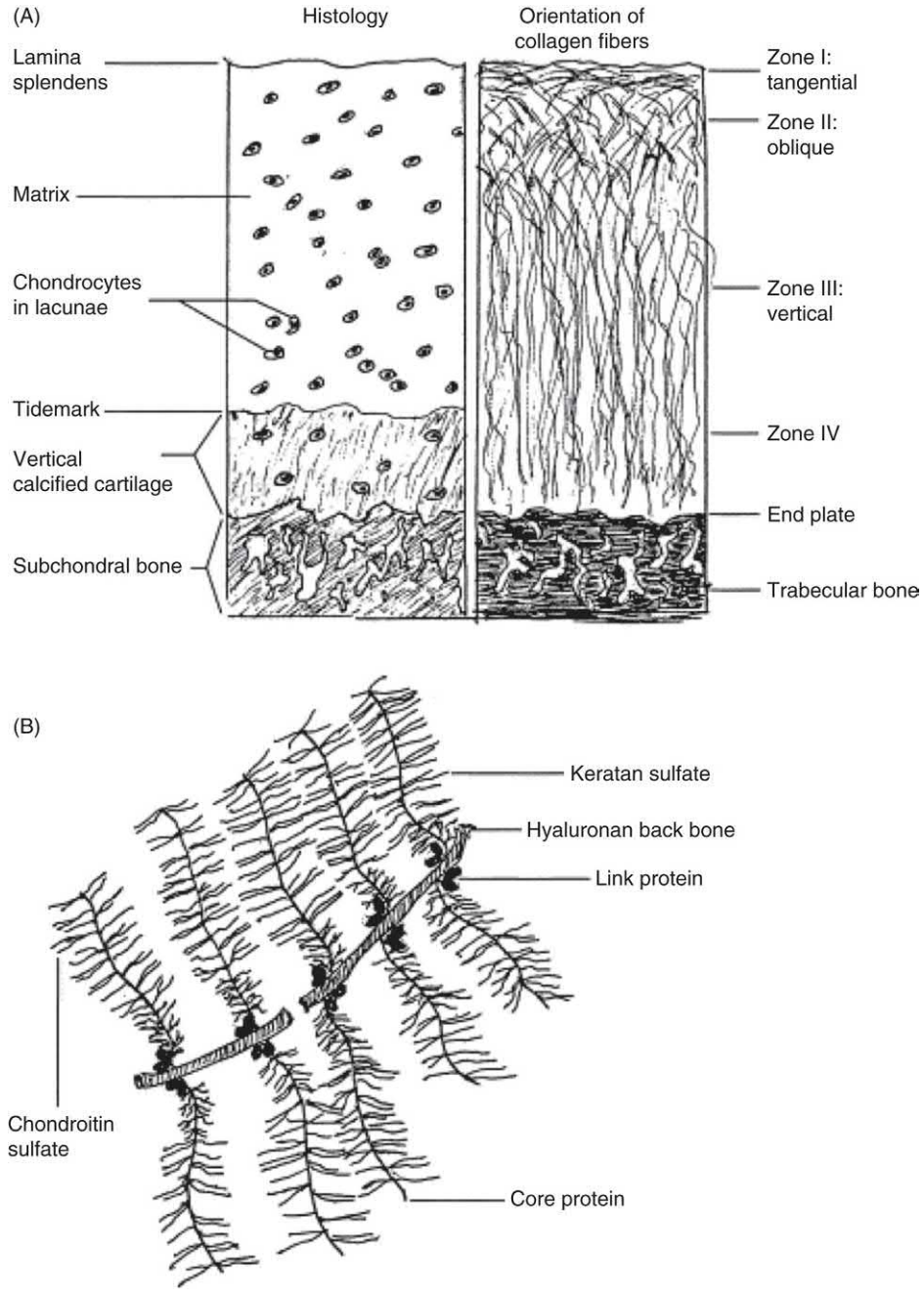

FIGURE 13.5 (A) The composition of articular cartilage includes chondrocytes and several ECM molecules. Chondrocytes only make up about 1% of the total volume of cartilage. The ECM contains two major types of molecules: collagen and proteoglycans. Over 90% of the collagen found in articular cartilage consists of type II collagen. This matrix protein provides much of the mechanical integrity of cartilage and comprises 60% of the total dry weight of cartilage. It is organized into fibers that compose four zones: Zone I or tangential, Zone II or oblique, Zone III or vertical, and Zone IV. Below Zone IV lie the end plate and the trabecular bone. Proteoglycans, on the other hand, compose 25%–35% of cartilage dry weight. (B) Aggrecans contain large amounts of chondroitin sulfate and keratan sulfate. Chondroitin sulfate and keratan sulfate, called glycosaminoglycans (GAGs), are modified with negatively charged sulfate groups and are highly polar. When many of these GAGs associate with a core protein to form a proteoglycan, the polar nature of the proteoglycans allows the molecules to interact strongly with water. *Source: Seal BL, Otero TC, Panitch A. Polymeric biomaterials for tissue and organ regeneration.* Mater Sci Eng *2001;***34***:147–230.*[96]

joint function and composed of three main components, such as collagens, proteoglycans, and other noncollagen proteins.[14,55] The articular cartilage is considered a low-turnover homeostatic tissue in which the chondrocytes support extracellular matrix synthesis and do not appear to proliferate in steady state conditions. The responses of the cells under pathological circumstances not only support the view that they can react to different stimuli including biochemical factors but also mechanical stress.[56] Maintenance of the ECM is necessary in particular in

response to changes in mechanical loading. Moreover, the network-like construction of the ECM molecules and the contact between its different components support the concept that the ECM is also a means of communication.[14,55]

3.1.1 Collagens

The major protein within the articular cartilage is collagen type II which forms the basis of a fibrillar network in association with other collagen fibrils, in particular collagen type XI[14,57] (Fig. 13.6). Collagens are trimeric molecules composed of three polypeptide chains. Collagen type II is a homotrimer with three α-1 chains that form the fibrils. The trimeric molecule is organized into a helix structure with every third amino acid being a glycine. The other amino acids are frequently prolines and hydroxyprolines and more specifically hydroxylysine in collagen type II. These specific residues are critical for the formation of oxidative crosslinks between the α-chains within one and between different collagen molecules. Crosslinking of different collagen molecules increases the resistance of the network against degradation by proteases. This protection is already established at a lower level by the helical structure and further increases with its complexity. Type II collagen also has nonhelical domains at its amino and C-terminal ends and these parts of the polypeptides play a critical role in its assembly process.

Type XI collagen in contrast is a heterodimer which forms fibrils in association with type II collagen. The combined fibrillar structure associates with type IX collagen at its fibrillar surface. Type IX collagen is a FACIT

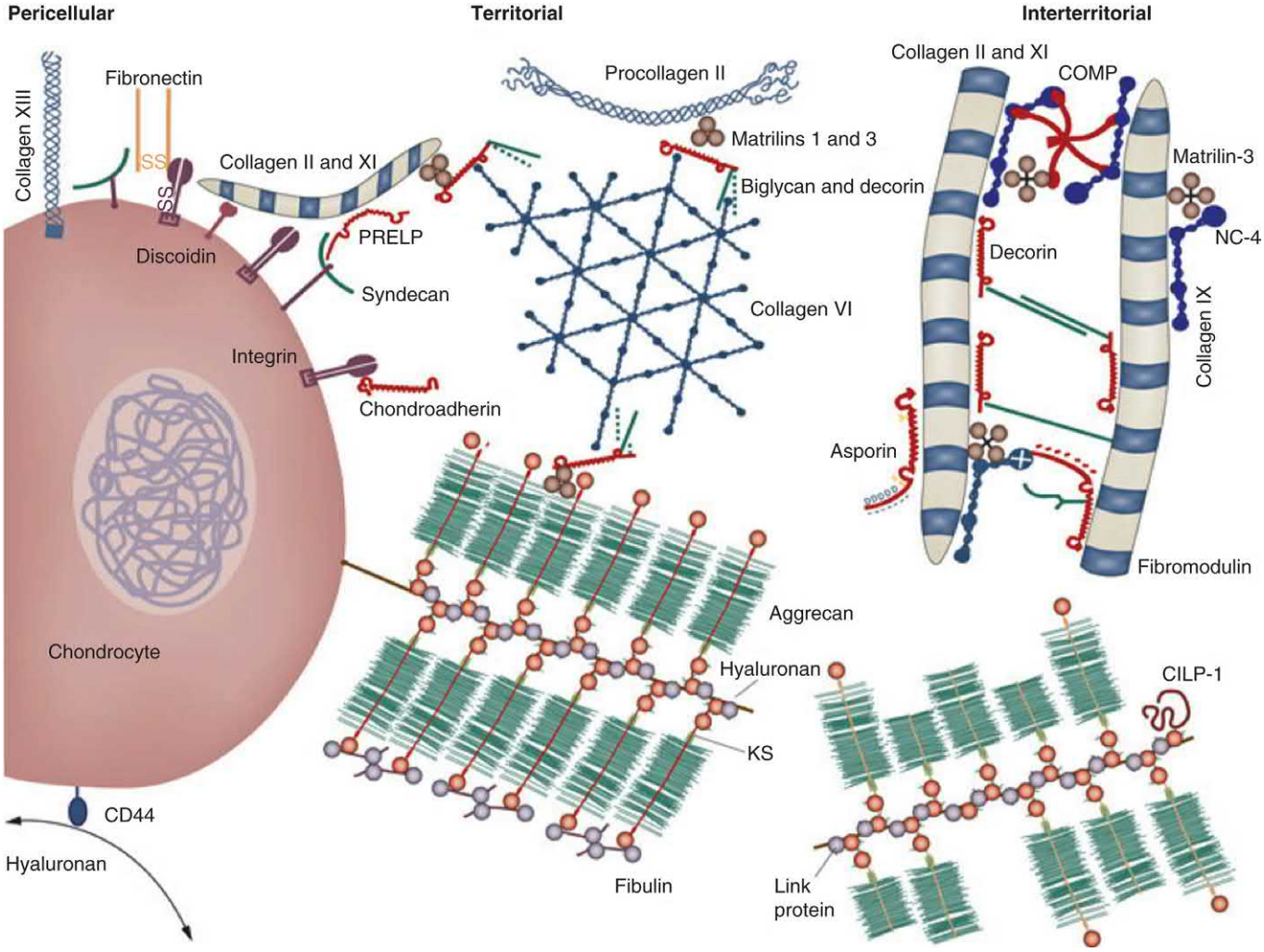

FIGURE 13.6 The cartilage matrix surrounding chondrocytes in healthy articular cartilage is arranged into zones defined by their distance from the cell. The pericellular matrix lies immediately around the cell and is the zone where molecules that interact with cell surface receptors are located; for example, hyaluronan binds the receptor CD44. Next to the pericellular matrix, slightly further from the cell, lies the territorial matrix. At largest distance from the cell is the interterritorial matrix. The types of collagens and the collagen-binding proteins that form the matrices are different in each zone. Abbreviations: CILP-1, cartilage intermediate layer protein 1; COMP, cartilage oligomeric matrix protein; CS, chondroitin sulfate; KS, keratan sulfate; PRELP, proline-arginine-rich end leucine-rich repeat protein. *Source: Seal BL, Otero TC, Panitch A. Polymeric biomaterials for tissue and organ regeneration.* Mater Sci Eng *2001*;**34**:*147–30.*[96]

collagen (fibril associated with interrupted triple helices). It consists of two collagen helix domains separated from a third helix by a noncollagenous domain. The third helix extends into the interfibrillar space together with a highly charged N-terminal domain allowing its interactions with other matrix proteins. The collagen network established by type II, type XI, and type IX collagens defines the interterritorial matrix within the articular cartilage.[57] This is the area found in between but not in the immediate proximity of the chondrocytes. Closer to the cells, the so-called territorial or pericellular matrix is different in its composition with the presence of a smaller network defined by type VI collagens.[58] Fibril formation is a complex process to which other ECM molecules contribute. These noncollagen proteins, discussed more extensively later, frequently remain attached to the collagen fibrils and further define the complexity of and potential interactions and communications within the matrix.

3.1.2 Proteoglycans

Proteoglycans are glycosylated proteins typically found in the ECM of connective tissues.[14,57,59] They can be distinguished from other glycoproteins by the presence of sulfated glycosaminoglycans (GAGs) bound to the core protein of the proteoglycan molecules. These sulfated GAGs are polysaccharides composed of disaccharide units characteristically containing repeats of a hexosamine and a hexuronic acid residue (Figs. 13.5 and 13.6). Three different types of sulfated GAGs can be distinguished, such as chondroitin sulphates, keratan sulphates and heparan sulfates. They are usually covalently linked to the core protein of the proteoglycan through O-glycosidase bonds.

Through their association with a large number of sulfated GAG side chains, proteoglycans are a rich source of negative charges within the ECM thereby attracting water molecules. These contribute to the articular cartilage's resilience and its reaction toward compressive forces. Under compression, water molecules are able to shift within the cartilage making the tissue softer and more flexible as compared to the subchondral mineralized bone. The proteoglycans are trapped in the earlier described collagen network which contributes to the specific structure and strength of the articular cartilage. The most abundant proteoglycan within the articular cartilage is aggrecan.

Hyaluronan is another macromolecule that belongs to the family of the GAGs but it is very different in its biological behavior.[60] It is not associated with the proteoglycan core as side chains, but forms a very large polymer and is not sulfated. Moreover, hyaluronan is synthesized outside the cell and is not assembled in the Golgi apparatus as other GAGs. Hyaluronan and aggrecan form very large aggregates making up the major molecular complex within the articular cartilage. The complex structure is built on the aggrecan molecules which contain three globular domains. The GAG side chains are found in between the second and third globular domain. The latter can associate with molecules, such as fibrillins, fibulins, and tenascin but its presence in the core protein is variable as different splice variants of aggrecan exist. The first globular domain is associated with hyaluronan and link protein in a ternary complex leading to the formation of macromolecules that together with the collagen network make up the most important part of the ECM.

3.1.3 Noncollagenous Matrix Proteins

The noncollagenous matrix proteins have important functions in the assembly of the matrix macromolecules, contribute to the complex network and thereby define properties and functions of the ECM.[14] Matrilin-3 is part of the matrilin family and a multimeric protein that can bind to triple helix collagens, biglycan, and decorin. It plays a role as a linker molecule in the network of the articular cartilage.[14]

The small leucine-rich proteins all have repeats of around 25 amino acids with leucine residues at conserved locations.[14] Decorin, biglycan, and asporin are typical examples of this group that bind collagens during fibril formation and thereby determine the rate and end of the process. They can also bind to the beaded head of collagen type VI molecules in the interfibrillar space. Fibromodulins, such as lumican bind to fibrillar collagens.[14] They are rich in sulfated tyrosine residues and acidic amino acids thereby forming a polyanionic domain that can also bind growth factors and cytokines and may determine local concentrations and effects of these signaling molecules.

Thrombospondins are a family of molecules of which cartilage oligomeric protein (COMP) is the best known.[14] COMP is a modular protein with a globular C-terminal domain with five subunits binding to collagen. It serves as a catalyst to bring collagen fibers together during their assembly. The balance between COMP and collagen fibrils may be very important for proper maintenance of the ECM. Detection of COMP in the serum of patients has been proposed as a biomarker for cartilage breakdown in joint diseases.

3.1.4 Organization of the Articular Cartilage

Within the human articular cartilage, a four-layered pattern can be distinguished[54,61,62] (Fig. 13.5). The cells of the superficial zone are lining the joint cavity. On top, the lamina splendens contains only fibrils and no cells. The cells underneath appear small and flattened with a relatively low amount of ECM surrounding them. Here, the ECM is rich in collagen but relatively poor in proteoglycans. These cells express lubricin and parathormone related peptide, which may play a role to keep them in their specific differentiation status. The organization of the ECM in the superficial layer confers its specific ability

to resist shear stresses and to serve as a guiding surface for joint movement. It may also limit the passage of molecules from the synovial fluid to the deeper layers of the cartilage. Cells in the middle zone or transitional layer appear more rounded and are usually dispersed as single cells throughout the ECM. These cells and matrix are involved in the transition between the shear forces found in the surface layer to the compression forces in the deeper cartilage layers. In the deeper radial layer, large cells are found in clusters and display a columnar organization in mammalian species. These cells typically express *Ihh* and *Runx2*. Below this zone, the articular cartilage becomes calcified. This transition can be recognized on histology by the tidemark, a basophilic transition line between both zones that is easily visible on microcopy sections.

3.1.4.1 Soft Tissues of the Joint

The *synovium* is a loose connective tissue that connects the opposing bones and lines the synovial cavity. The lining layer is, under normal circumstances, an epithelial-like structure without basement membrane. Two types of cells are found within the lining layer: type A synoviocytes belongs to the macrophage lineage and type B synoviocytes also described as synovial fibroblasts.[63] The architecture of the lining layer appears to be dependent on adhesion molecule cadherin-11.[51] The cells of the lining layer produce major components of the synovial fluid, such as lubricin and hyaluronan. Lubricin attracts an additional layer of water molecules on the edges of the articular cartilage smoothening the movement of the opposing sides against each other. Hyaluronan protects the synovium from being locked and pinched in between the cartilage. The synovial sublining zone is a loose connective tissue with blood and lymphatic vessels, some fibroblasts, pericytes, and a small amount of cells belonging to the immune system. The vessels are very important to bring nutrients to the synovium and the joint cavity as the nourishment by diffusion of the articular cartilage is largely dependent upon it. The synovium has the potential to attract massive amounts of cells under inflammatory circumstances and plays a key role in chronic arthritis. There is a large heterogeneity in the sublining zone, such as characteristic loose connective tissue, more dense fibrous zones, and also fat pads. The latter may be specifically involved in inflammation in joint diseases.[64]

Some joints, such as the knee joints contain additional fibrocartilaginous structures, such as menisci. These are important to support motion in the specific joints. Ligaments, capsules, and tendons are composed of dense connective tissue and particularly rich in type I collagen fibers which confer their tensile strength.

3.1.4.2 The *Enthesis*

Ligaments, capsules, and tendons insert into the underlying bone in a specific anatomical zone called the *enthesis*.[65] The *enthesis* is a multilayered structure important in translating mechanical stress triggered by movement from the tendons or ligaments toward the underlying bone. It consists of an uncalcified fibrocartilage layer through which the collagen fibers of tendon or ligaments are passing, followed by a calcified fibrocartilage layer and then inserting onto the bone. The tidemark between the uncalcified and calcified cartilage is relatively straight. The transition between fibrocartilage and underlying bone is irregular thereby increasing the surface of the interaction and providing additional strength. Like the articular cartilage, the *enthesis* is largely nonvascular and resistant to cell invasion. Tissue responses toward entheseal overload or damage are therefore hypothesized to accrue cells into the adjacent synovium and bone marrow with which the *enthesis* is connected through small channels in the bone. This so-called synovio–entheseal complex is hypothesized to play key roles in some chronic joint diseases, such as ankylosing spondylitis and psoriatic arthritis.[66]

4 JOINT HOMEOSTASIS

Proper joint function is critically dependent on the complex maintenance of homeostasis in all joint tissues.[67] The articular cartilage needs to maintain its resilience and relative softness, with the synovium providing nutrients and lubrication. From the biomechanical perspective, the cartilage–bone interface appears of particular importance.[56] Both the chondrocytes as well as the bone cells found in the subchondral zone perceive strain and load and will react to these stimuli. Maintenance of homeostasis is likely based on a specific balance between the resistance of the mineralized tissues and the flexibility of the softer articular cartilage. Moreover, the interface between cartilage and subchondral bone has recently been associated with intense molecular crosstalk between the tissues. The existence of vascular communication channels and the irregular border between the tissues form a junction over which diffusion of small molecules can occur. Not only is the border between the deeper calcified layers of the articular cartilage and the bone irregular but also patches of uncalcified cartilage can come into contact with the bone facilitating molecular interactions. Changes in this system likely contribute to the onset and progression of joint diseases.[56]

5 JOINT DISEASE

Chronic joint diseases are among the most frequent health problems and have a high burden on society due to morbidity, disability, and high-socioeconomic costs.[68] The most common joint diseases are osteoarthritis (OA),

rheumatoid arthritis (RA), and spondyloarthritis. The latter two are chronic inflammatory diseases linked to autoimmunity and autoinflammation respectively. OA is too often considered as a simple degenerative disease or a natural consequence of aging but should be considered as a more complex disorder to which both genetic and acquired factors contribute ultimately leading to joint failure.

5.1 Rheumatoid Arthritis

RA is a chronic inflammatory systemic disease mainly affecting the synovial joints and associated with autoimmune phenomena, such as autoantibodies including the so-called rheumatoid factor and anticitrullinated protein antibodies.[69] The key role of these autoantibodies in onset and course of the disease support the view that RA is an autoimmune disease. RA affects more women than men and usually occurs between the third and fifth decade of life. The disease susceptibility is determined by genetic factors but environmental factors, such as smoking also play an important role in the onset of disease. Patients experience joint paint, swelling, and loss of function. RA typically symmetrically not only affects the small joints of hands and feet but may also involve larger joints of the peripheral skeleton. Involvement of the spine is very rare with the exception of the articulation between the dens and axis, the first two cervical vertebrae. The clinical picture of arthritis is caused by synovial inflammation and joint effusion. The inflammatory process leads to progressive cartilage and bone destruction (Fig. 13.7). The synovium undergoes a dramatic transformation with the synovial fibroblasts proliferating and acquiring an "aggressive" phenotype.[63] They produce tissue destructive enzymes, such as matrix metalloproteinases and other proteases breaking down the ECM of the articular cartilage. Activation of synovial fibroblasts and T cells triggers the RANKL–RANK cascade and stimulates the differentiation, maturation, and activation of osteoclasts leading to juxtaarticular bone erosions.[70]

A number of therapeutic options are available.[69] The introduction of targeted biological therapies directed against cytokines, such as tumor necrosis factor or interleukin-6, or against immune cell populations, such as T and B cells, has greatly improved the outcome of the disease in a large number of patients. Strategies are aimed at controlling inflammation and preventing structural damage, and in RA both these processes are linked to each other. Therefore early disease detection becomes a major point of attention.[71] Successful treatment of the disease with either potent immune modulators and increasingly with targeted biological therapies is capable of controlling symptoms as well as preventing damage to the joints.

5.1.1 Spondyloarthritis

The spondyloarthritis concept groups different diagnostic entities that share clinical, genetic, and pathological characteristics.[72] Within this concept ankylosing spondylitis, psoriatic arthritis, reactive arthritis, and arthritis associated with inflammatory bowel disease are the best-known subforms. In addition undifferentiated and juvenile spondyloarthritis are also recognized. The clinical presentation of spondyloarthritis can therefore be very different between patients. In contrast to RA which is characterized by a symmetrical peripheral polyarthritis, spondyloarthritis affects the sacroiliac joints and the spine. Peripheral joint involvement, if present, is mainly nonsymmetrical and affects the lower limbs. However, polyarticular forms do exist. New concepts integrating evidence from magnetic resonance imaging, distinguish a mainly axial pattern of disease, in which the spine and

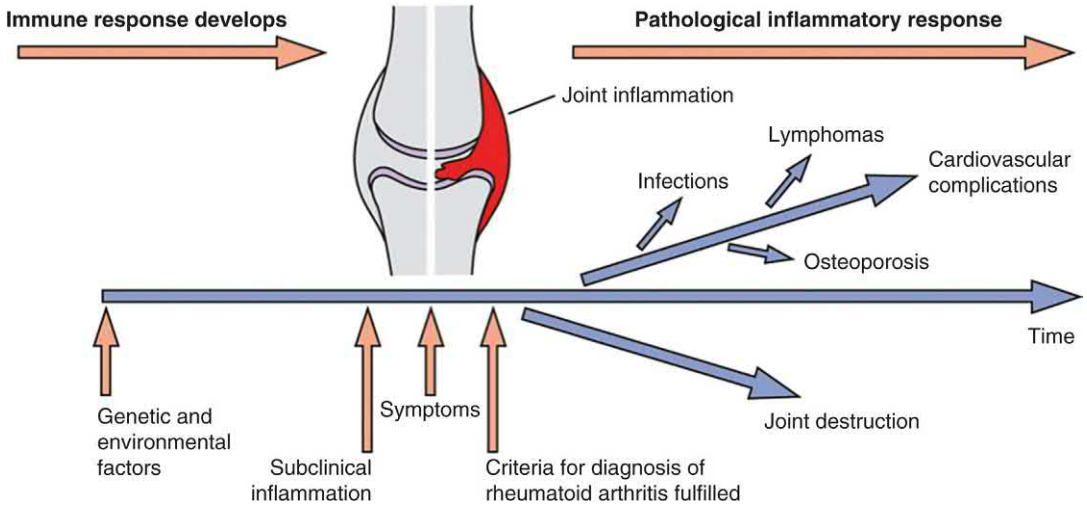

FIGURE 13.7 Longitudinal course of rheumatoid arthritis from preclinical stages to joint damage and systemic manifestations of the chronic inflammatory joint disease. *Source: Klareskog L, Catrina AI, Paget S. Rheumatoid arthritis.* Lancet *2009;***372***(9664):659–72.*[69]

sacroiliac joints are affected, from a predominantly peripheral disease.[73] In spondyloarthritis patients, inflammation as manifested by osteitis, enthesitis, and synovitis, triggers pain and loss of function. There is a strong genetic association with the HLA-B27 gene and novel genetic factors have recently been identified.[74] There is a little evidence that acquired immune responses play any role in the pathogenesis.[75] Moreover, tissue damage and structural progression of disease are characterized by new bone formation leading to spine or joint ankylosis instead of joint destruction.[75] Treatment strategies for spondyloarthritis are limited but the introduction of anti-tumor necrosis factor drugs has greatly changed the perspective and prognosis of the patients. Many challenges in spondyloarthritis remain, in particular the prevention of long-term structural damage caused by progressive ankylosis, a process that appears molecularly uncoupled from inflammation and for which current drug strategies do not have a short-term benefit.

5.1.2 Osteoarthritis

The osteoarthritic diseases cause the most common chronic joint problems. OA may affect not only single or multiple joints, including large joints, such as the hip or knee, but also the small joints of the hands and the spine. Although most commonly found in the elderly, the disease process is complex and both genetic and acquired factors may contribute to its onset, course ,and outcome.[76,77] Abnormal loads on a normal joint as well as normal loads on a structurally abnormal joint may contribute to the prevalence and progression. OA is characterized by progressive degradation of the articular cartilage, thickening of the subchondral bone, and the appearance of bone outgrowths on the edges of the joint called osteophytes (Fig. 13.8). Progression of disease is also associated with synovial inflammation and increasing pain.

OA is an interesting case study on failure of joint homeostasis. In patients that develop OA, homeostatic and repair mechanisms do not match the catabolic events that are the consequence of stress and strain in the chondrocytes and the subchondral bone.[56] In response to these stresses, not only chondrocytes increase their metabolism and ECM synthesis but also matrix turnover with a rise in tissue destructive enzymes, such as matrix metalloproteinases and aggrecanases. When the catabolic cascades outweigh the anabolic potential, cartilage destruction is the net result. In parallel, subchondral bone cells are activated and increase their matrix synthesis. This results in apparent thickening or sclerosis of the subchondral bone. Increased bone anabolism is paralleled by increased osteoclast activation resulting in bone breakdown. As the mineralization process cannot match the speed of matrix synthesis, the net result here is a thicker but weaker and less structured bone also contributing to progressive joint failure. Therapeutic pharmaceutical opportunities in OA are limited to painkillers and anti-inflammatory drugs and at the end stage of the disease prosthesis or joint-corrective surgery is often required.[76]

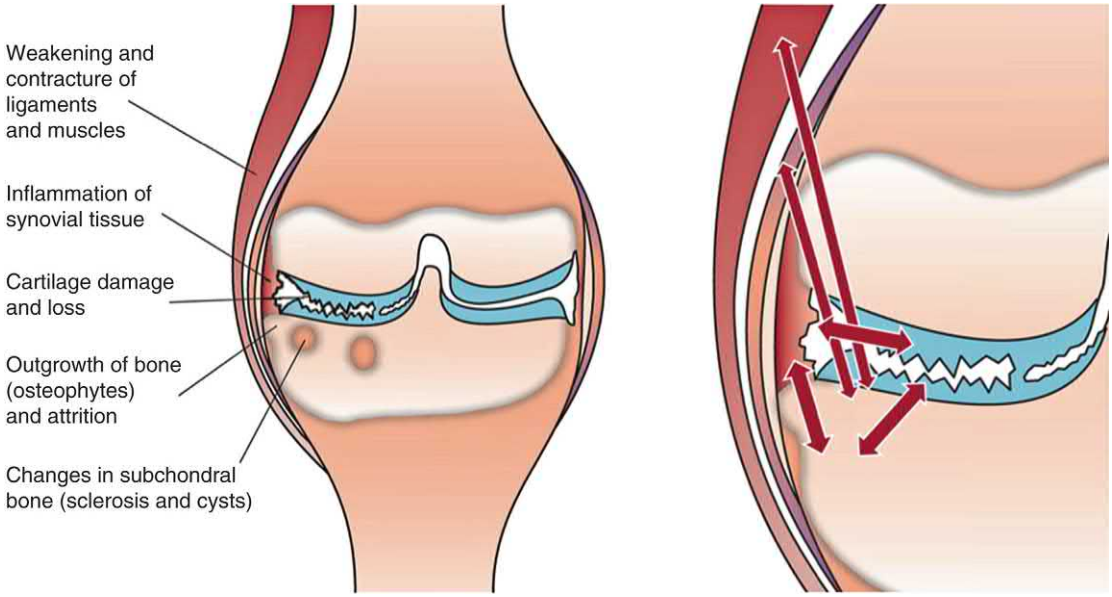

FIGURE 13.8 Schematic drawing of an osteoarthritic joint. The different tissues involved in clinical and structural changes of the disease are shown on the left. Note that cartilage is the only tissue not innervated. On the right the bidirectional interplay between cartilage, bone, and synovial tissue involved in osteoarthritis is shown, and the two-way interaction between this interplay and the ligaments, and muscles. In the interplay between cartilage, bone, and synovial tissue one of the tissues might dominate the disease, and as such should be targeted for treatment. *Source: Bijlsma JWJ, Berenbaum F, Lafeber PJG. Osteoarthritis: an update with relevance for clinical practice.* Lancet *2011;***377***(9783):2115–26.*[76]

6 JOINT REPAIR

As the lifespan of prosthetic devices is limited, new treatment approaches to repair skeletal structures, such as the joints need to be devised for the young- and middle-aged individuals with joint damage caused by congenital, traumatic, or inflammatory conditions. It is believed that joint repair through regenerative medicine and more specifically tissue engineering approaches may fill this void to some extent.[78]

6.1 Tissue Repair by Enhancing the Endogenous Healing Response

For interventional tissue repair, two mechanistic approaches are possible: enhancing intrinsic repair mechanisms including stimulation of cell proliferation, differentiation, and metabolic activity of the tissue, and the recruitment of endogenous progenitor populations into the damaged tissue. Extrinsic repair is the alternative if insufficient intrinsic repair leads to clinical symptoms and signs, with loss of function. This includes tissue engineering approaches using cell populations and combination products of cells, matrices, and scaffolds that can contribute mostly locally to the tissue repair processes. Some of these targeted anabolic and tissue repair approaches to stimulate intrinsic repair have been translated into the clinics with, for example, OP1/BMP7[79] and fibroblast-like growth factor[80] being explored in early phase clinical trials for OA and joint surface defects.

6.2 Joint Resurfacing by Tissue Engineering

Cell-based therapies and tissue engineering for joint repair are starting to enter the clinical arena, and thus becoming of relevance to the clinicians today. Many preclinical and early clinical applications appear promising. This relates particularly to the use of autologous and allogenic adult MSCs in musculoskeletal tissue repair and regeneration in general, and joint disorders/disease and OA more in particular.[78] MSCs are defined by their self-renewal capacity and their ability to differentiate in vitro into chondrocytes, osteoblasts, or adipocytes.[81] The mechanisms through which the cellular therapies and combination products in vivo can contribute to tissue repair and interfere with disease processes are multiple and involve not only the direct engraftment into damaged tissue, proliferation, and differentiation to tissue specific cell types, but also include paracrine actions, such as the secretion of growth and differentiation factors that enhance the local tissue responses toward the damage that has occurred. Indeed, cellular products can be considered multisignal delivery systems and interact with the microenvironment.[82] This is obviously a strength of the approach but it also highlights the complexity of developing a cell therapy and the need for control over quality, consistency, and safety of such products. Indeed, cellular products are now subjected to regulatory review and approval before they can enter clinical practice. Cell-based products belong to the new class of Advanced Therapy Medicinal Products regulated by the European Medical Agency.

Some tissue engineering applications have reached the clinic and aim to restore the integrity and function of the joint and in particular the joint surface. Among them and most prominently, are treatments for the repair of articular cartilage and osteochondral defects in otherwise healthy joint or (early) OA. Marrow stimulation techniques were described for the first time by Pridie in 1959. A cartilage lesion was debrided back to stable borders and the subchondral plate was perforated with a drill.[83] The microfracture method developed out of this tradition and is most widely used and is still considered a first-line treatment for small focal cartilage defects in joints, primarily in the knee.[84] It is technically an easy procedure that can be performed via arthroscopy. Although the repair tissue is a mixture of fibrous and cartilaginous tissue with the presence of some collagen type II and proteoglycans, this tissue tends to degenerate over time into fibrous tissue resulting in recurrence of clinical symptoms and signs.[85,86] Marrow stimulation techniques may however be less suitable, or at least may be more complex for the treatment of OA, as the subchondral bone and bone marrow characteristics are profoundly affected in this disease, and thus the biology will be quite different and to be explored.[56]

Regeneration or repair of symptomatic articular cartilage defects has been on the forefront of regenerative medicine ever since Brittberg et al. reported in 1994 a remarkably good clinical and structural outcome using a procedure called autologous chondrocyte transplantation/implantation (ACI).[87] Briefly, cell populations were prepared by enzymatic release from a biopsy of articular cartilage taken from an unloaded area in the symptomatic joint during arthroscopy. The chondrocytes were subsequently expanded in vitro, and reimplanted through arthrotomy in the joint surface defect under a periosteal flap, taken from the tibia from the same patient. This was then followed by a rehabilitation to reach its optimal outcome at 18–24 months. Since then, progress has been made by improving and standardizing the preparation of the autologous chondrocytes, the development of other delivery systems for the chondrocytes and the replacement of the periosteal flap by a membrane of diverse composition, and a series of prospective multicenter clinical studies. Recent trials strongly suggest that ACI with a well-defined cell product and an applied rehabilitation protocol may be superior in the long term to microfracture.[88,89] Although still open for debate with respect to long-term clinical durability, there is a broad

consensus that ACI can be considered as a regenerative treatment with good structural and clinical outcome. Importantly, the data indicate that the outcome may be superior to standard care if the "proper" patients are treated. Positive predictors of good outcome include early intervention (less than 3 years symptomatic), good quality of chondrocytes, well-trained surgeons, adherence to rehabilitation protocols, and no signs of OA (as defined by Kellgren II). Cost-effectiveness will certainly improve if the treatment is also shown to prevent the progression toward OA, but these data are not yet available.

The so-called "second generation" ACI, cells cultured in/on a matrix might have some distinct advantages over the "classical" cell suspensions implanted under a periosteal flap. Culturing the cells in 2D-plus (in multilayer on a membrane) or 3D-conditions (distributed in a matrix) seems to favor the phenotypic stability of the chondrocyte.[90,91] Due to the fact that suturing is not always required, the surgery becomes easier and can be performed through a mini-open or even arthroscopic procedure.[92] Recent data report superiority of this approach after 2 years over microfracture in a prospective randomized trial.[93] At this moment, several matrices are in use in a clinical setting, but it is unclear which is the ideal matrix at this time. The new wave of developments within this field relate to the use of autologous and/or allogenic progenitor cell populations for the repair of joint surface defects in combination with new biomaterials, and the application of gene therapy.[94] Recent evolutions also study the use of noncellular approaches including nano-scale scaffold structures. Although some data in preclinical models appear of interest, applications in human are still in their first steps.

In conclusion, tissue repair approaches so far have mostly been investigated in damaged posttraumatic joints. No or very limited data have been reported with these tissue engineering approaches in established OA. Indeed, as the microenvironment in OA is very different when compared to damaged joints without signs of definite OA, we cannot extrapolate the data to (osteo)arthritic disease. Recent developments identify the potential of MSCs and their secretory vesicles in the treatment of OA, with promising results in preclinical animal models of OA and early phase clinical trials in men (for review).[95]

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CHAPTER

14

Osteocyte Biology

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1 INTRODUCTION

Osteocytes are the most abundant cells in bone and play a central role in the execution of the functions of the skeleton. At one time thought as inactive quiescent cells buried in the mineralized tissue, osteocytes coordinate bone acquisition during growth and are critical for the maintenance of a strong and healthy skeleton able to accomplish the functions of locomotion and protection of essential organs. Osteocytes regulate the genesis and activity of osteoblasts and osteoclasts through factors produced and secreted in response to mechanical and hormonal stimuli, thus adapting the skeleton to changes in environmental cues. In addition, osteocytes contribute to the endocrine functions of bone by secreting hormones that affect other tissues; and they also regulate mineral homeostasis and hematopoiesis, which are essential for organismal life. The central role of osteocytes was long foreseen by pioneers in the field, who predicted that these cells contribute to skeletal homeostasis. Booming research on the biology of osteocytes of the last 2 decades unraveled some of the mechanisms underlying the functions attributed to osteocytes, recently reviewed.[1–3] It is envisioned that the intense current research will continue to increase our knowledge of osteocyte biology and pathophysiology.

2 THE FUNDAMENTAL ROLE OF OSTEOCYTES IN SKELETAL HOMEOSTASIS

2.1 The Osteocyte Network

The intricate network that connect osteocytes with each other and with cells on the bone surface and the vasculature has fascinated bone biologists for long time. The studies by Marotti and coworkers using microscopy of human bones showed that osteocytes within lacunae extend multiple cytoplasmic projections reaching neighboring osteocytes and cells on bone surfaces.[4] Based on these morphological features, these investigators hypothesized that osteocytes are central players in a cellular network in which cells are connected via gap junctions and are able to sense mechanical and biochemical signals.[5] Knowledge accumulated during the last few years supports the important role of the osteocyte network for skeletal homeostasis and has identified mechanisms and messengers by which osteocytes control not only bone formation, but also resorption. Recent evidence now supports a role for the osteocyte network in the distribution of molecules, such as sclerostin, osteoprotegerin (OPG), and fibroblast growth factor 23 (FGF23), which reach cells on the bone surface, the bone marrow (BM), and even the general circulation (Fig. 14.1). Premature death of osteocytes and disruption of the osteocyte network is likely to negatively impact the skeletal response to environmental cues (as will be discussed later in Section 6).

2.2 Osteocytes as Mechanosensory Cells

The skeleton adapts to meet mechanical needs by changing its mass, shape, and microarchitecture.[6–8] Frost developed the mechanostat theory that proposes that the magnitude of the mechanical stimulation applied to bone dictates whether bone will be increased (by increasing bone formation) or reduced (by increasing bone resorption).[9] In this model, osteocytes sense the load imposed to bone and respond by signaling to osteoblasts and osteoclasts to adapt to mechanical changes.[10] Osteocytes are proposed to act as mechanosensors.[11] Whereas bone-forming osteoblasts and bone-resorbing osteoclasts

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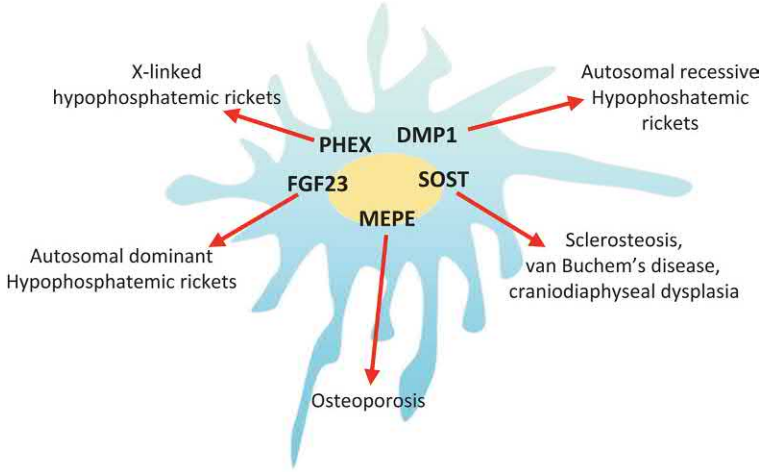

FIGURE 14.1 In addition to normal physiological functions, osteocyte factors can also play a role in bone and kidney disease. Mutations in the early osteocyte factors PHEX and DMP1 can play a role in hypophosphatemic rickets as does FGF23. The mature osteocyte factor MEPE plays a role in osteoporosis and the mature osteocyte factor, sclerostin, coded by the gene SOST, plays a role in high bone mass genetic disease.

are present on bone surfaces for relatively short periods of time and in low numbers, osteocytes are by far the most abundant resident cells and are present throughout the entire bone tissue. Their abundance and strategic location in the core of the lacuna–canalicular network make these cells the most suitable candidates for detecting variations in the level of strain and for distributing signals leading to adaptive responses.[12] However, the molecular mediator(s) had remained elusive until it was demonstrated that mechanical loading decreases the expression of Sost/sclerostin in osteocytes, and that conversely, unloading increases it. Moreover, areas of cortical bone receiving greater strain stimuli and higher bone formation exhibit a greater reduction in both the number of sclerostin-positive osteocytes and the intensity of sclerostin staining per osteocyte.[13] These findings support the notion that osteocytes coordinate the osteogenic response to mechanical force by downregulating sclerostin, thereby locally unleashing the powerful Wnt anabolic signaling.

Indeed, the osteogenic response to mechanical stimulation is disrupted in mice overexpressing in osteocytes a human SOST transgene that cannot be downregulated by loading (DMP1-SOST).[14] Thus, WT mice showed the expected decrease in sclerostin expression in osteocytes and a robust and dose-dependent increased bone formation upon ulnae loading, DMP1-SOST mice exhibited an overall reduced sensitivity to mechanical stimulation. Moreover, loading increased the expression of several genes associated with activation of Wnt signaling in wild type (WT) mice, whereas this effect was absent in DMP1-SOST mice.[14,15] Thus, Sost/sclerostin downregulation is an obligatory step for mechanotransduction, and for activation of the Wnt pathway and the increase in bone formation induced by mechanical force. Furthermore, other osteocyte-derived molecules, including RANKL and the Wnt target gene OPG and are also involved in bone adaptation to mechanical stimuli.[11]

2.3 Osteocytes and the Skeletal Response to PTH

Parfitt proposed in the 1970s that osteocytes were involved in the response of the skeleton to parathyroid hormone (PTH).[16] Based on the rapid timing of hypercalcemic responses to PTH, the fact that new protein synthesis is not required, and that calcium is released into the circulation at two different rates, Parfitt concluded that osteocytes control the rapid release of calcium, whereas osteoclastic resorption determines the later phase of calcium released from bone to the circulation induced by PTH. Studies using genetically modified mice have recently demonstrated profound skeletal effects of activation of the PTH receptor in osteocytes[17–20] and defective response to PTH of mice lacking this receptor in osteocytes.[21,21a] These studies support and extend Parfitt's concept that indeed osteocytes are target cells of PTH action. Further, mice lacking the PTH receptor in osteocytes lose less bone with lactation, a condition in which calcium release from the skeleton to the blood induced by PTHrP (the other ligand of the PTH receptor) is required for milk production in the mammary glands.[22] In addition, the characteristic increase in osteocyte lacunar size observed in lactating wild-type mice is not found in mice, lacking the PTH receptor in osteocytes, suggesting that removal of perilacunar bone by osteocytes as a source of calcium contributes to the bone loss. The process of perilacunar remodeling, named earlier by Belanger osteocytic osteolysis,[23] is achieved by the expression in osteocytes of gene usually expressed by osteoclasts during bone resorption. These genes encode for proteins that decrease extracellular pH, such as carbonic anhydrases and ATPases, that degrade

the extracellular matrix, such as MMP13 and cathepsin K, and that dissolve the mineral, such as TRAPase.

3 MOLECULAR AND FUNCTIONAL SIGNATURE OF OSTEOCYTES

Between 5% and 20% of the osteoblasts present on the bone surface become surrounded by matrix proteins that they produce and differentiate into osteocytes,[8,24] by mechanisms that are still uncertain. This transition is characterized by alterations in gene expression and it is accompanied by morphological and functional changes. Osteoblasts are highly secretory cells characterized by cuboidal shape, large nucleus located close to the cell basal membrane, enlarged Golgi apparatus on the nuclear apical surface, and extensive endoplasmic reticulum. During osteocytogenesis, the number of cellular organelles markedly reduces and the ratio between nuclear and cytoplasm volume increases as the cell acquires a star-like morphology. The long osteocytic cytoplasmic processes run through canaliculi, "dug" in mineralized bone, and touch neighboring osteocytes, bone surface cells, and endothelial cells of the blood vessels. This extensive lacunar–canalicular system is maintained by the ability of osteocytes themselves to remodel their surrounding space, as demonstrated by calcification of the lacuna and reduced numbers of canaliculi when osteocytes are lost by apoptosis.

3.1 Formation and Maintenance of the Osteocytic Network

The transition from osteoblasts to osteocytes occurs while the cells become surrounded first by unmineralized matrix or osteoid, and later, by mineralized bone matrix. Several molecules might mediate the development of dendrites by dictating the formation of osteocytic projections or, indirectly, by controlling mineralization and matrix degradation and thus the formation of the lacunar–canaliculi system.

E11 (also called OTS-8, podoplanin, gp38, or T1α), originally described in osteoblastic cells and subsequently cloned from other tissues,[25,26] is expressed in dendritic cells of the kidney, lung, brain, and skin.[27,28] In bone, E11 is expressed in newly embedded osteocytes, but not in more mature osteocytes surrounded by mineralized bone matrix or in osteoblasts on the bone surface, indicating that is a marker of early osteocyte differentiation.[29] E11 expression is required for dendrite elongation induced by loading in vitro and it is increased by mechanical stimulation in vitro and in vivo, suggesting that mechanical signals might control osteocytogenesis. E11 binds to CD44, a molecule also expressed in neuronal dendrites,[30] and expression of either molecule has been associated with osteocytic dendrite branching.[31,32] Osteocytic dendrites also contain α-actinin and fimbrin, actin-bundling proteins required for the cytoskeletal organization of osteocytes isolated from chicken.[33,34] Mutations of PLS3, the human homolog of fimbrin that encodes for the protein Plastin 3, causes X-linked osteoporosis with fractures,[35] suggesting an association of deficient dendrite formation with bone fragility.

Dentin matrix protein 1 (DMP1) is expressed in mature osteoblasts and its expression increases as osteoblasts differentiate toward osteocytes.[36] DMP1 expression is required for proper osteocyte maturation, as evidenced by abnormally elevated expression of osteoblastic and early osteocytic genes (such as alkaline phosphatase, collagen type 1 and E11) and low expression of sclerostin (a marker of mature osteocytes) in deeply embedded osteocytes in mice lacking the DMP1 gene.[37] Mineralization is also defective in DMP1 knockout mice likely causing the incomplete differentiation of osteocytes and the disorganized osteocytic lacunar–canalicular system. Interestingly this can be corrected by normalizing phosphate levels using anti-FGF23 antibodies[38] or by improving bone mineralization with antisclerostin antibodies.[39] As for DMP1, the expression of matrix metalloproteinases (MMPs) also increases as osteoblasts differentiate into osteocytes. The function of MMPs in osteocytogenesis might be related to their ability to cleave collagen in the matrix surrounding osteocytes, allowing the formation of canaliculi through which osteocytes extend cytoplasmic projections. Consistent with this notion, mice lacking MMP14, a member of the MMP family of proteins, exhibit reduced or absent osteocytic processes.[40] Further, the ability of collagen to be degraded by MMP13 is required to maintain osteocyte viability, as demonstrated by increased osteocyte apoptosis in mice expressing a mutated nondegradable collagen 1.[41]

A molecule crucial for the functionality of the osteocyte network is connexin 43 (Cx43), the most abundant member of the connexin family of proteins expressed in osteocytes.[42] Cx43 forms gap junction channels between cells participating in cell-to-cell communication within the osteocyte network; and it also forms hemichannels connecting cells with the extracellular milieu. Deletion of the Cx43 gene from osteocytes in mice decreases osteocyte viability and induces changes in the geometry of long bones, which present the features of bones from old mice or humans.[43–45] Apoptotic osteocytes and empty lacunae are increased, the BM cavity is enlarged by exaggerated endosteal resorption, and the diameter of the bones is larger due to increased periosteal bone apposition. These effects of Cx43 deletion are intrinsic to osteocytes, as knockdown of the gene in vitro result in cells that die spontaneously and exhibit increased RANKL/OPG ratio and increased ability to support osteoclast formation.[45,46] Further, osteoclasts are found on surfaces

adjacent to areas where apoptotic osteocytes accumulate in Cx43 knockout mice, suggesting that signals released by dying osteocytes are required to target osteoclast recruitment. Cx43 expression in osteoblasts/osteocytes is also required for cell survival induced by bisphosphonates or PTH, as well as for a full bone anabolic response to PTH.[47–50] Cx43 is also involved in the response of osteocytes to mechanical signals. However, PGE_2 release induced by in vitro mechanical stimulation requires Cx43 expression in osteocytes, paradoxically Cx43 restrains the response of bone to mechanical stimulation in vivo as deletion of the Cx43 gene from osteocytes enhances the anabolic response of bone to loading.[51–53] This suggests that Cx43 does more than regulate PGE_2 release in vivo. Future research is warranted to explain the mechanisms underlying this phenomenon.

3.2 Regulation of Bone Formation by Osteocytes

One of the most important events of the last 2 decades that advanced our understanding of skeletal biology was the discovery of the role of Wnt/βcatenin signaling in bone.[54] The so-called canonical Wnt-signaling pathway controls fate of mesenchymal stem cells and their differentiation into the various cell lineages. Canonical Wnt signaling restrains chondrogenic and adipogenic differentiation while enhancing osteoblastic differentiation from mesenchymal stem cells. In addition, Wnt/βcatenin signaling promotes osteoblast maturation and survival of osteoblasts and osteocytes. At the same time, it inhibits osteoclastogenesis, although indirectly by increasing the expression of OPG in osteoblasts and osteocytes.

Osteocytes are critical players in the regulation of this signaling pathway both as targets of the Wnt ligands and as producers of molecules that modulate their action. Wnt/βcatenin signaling is activated by binding of Wnt proteins to a receptor complex composed of frizzled receptors and coreceptors of the low density lipoprotein receptor–related protein (LRP) family, LRP5 and 6. This event stabilizes βcatenin, induces its translocation to the nucleus, and activates gene transcription. Activation of this pathway is critical for bone acquisition and maintenance through increased bone formation and decreased resorption.

Dickkopf-related protein 1 (Dkk1) is a Wnt antagonist expressed in osteoblasts and at higher levels in osteocytes.[55] Dkk1 binds to LRP5/6 preventing the binding of Wnt ligands. Mice overexpressing Dkk1 in osteoblastic cells exhibit decreased bone formation and low bone mass, consistent with the stimulatory role of the Wnt/βcatenin pathway on bone formation.[56] Another Wnt inhibitor, secreted frizzle-related protein 1 (sFRP1) is expressed in early osteocytes and its levels decrease as osteocytes mature.[57] Consistent with the role of sFRP1 as an inhibitor of anabolic Wnt/βcatenin signaling, its deletion from the mouse genome results in increased bone mass.[54]

Another antagonist of Wnt signaling is sclerostin, a protein encoded by the Sost gene, which is primarily expressed by mature osteocytes but not by early osteocytes or osteoblasts.[58] Sclerostin binds to the Wnt coreceptors LRP5/6 antagonizing downstream signaling.[59] Sclerostin also binds to LRP4, another member of the LRP family of proteins, which acts as a chaperone and is required for the inhibitory action of sclerostin on Wnt/βcatenin signaling.[60] Absence of sclerostin expression or secretion in humans causes sclerosteosis, van Buchem disease, or craniodiaphyseal dysplasia, inherited high bone mass (HBM) conditions characterized by exaggerated bone formation.[54] Further, inactivating mutations of the Wnt coreceptor LRP5 result in low bone mass (osteoporosis pseudoglioma); and, conversely, activating LRP5 mutations lead to HBM partially due to decreased binding affinity of the mutated LRP5 for sclerostin.[54] Consistent with the requirement of LRP4 for the inhibitory function of sclerostin, individuals with LRP4 inactivating mutations exhibit HBM. Moreover, deletion of the Sost or LRP4 genes in mice or neutralizing antibodies for sclerostin or LRP4 reproduces the HBM phenotype found in humans lacking SOST or LRP4 activity,[61–65] whereas overexpression of Sost/sclerostin decreases bone mass.[14,18,66,67] Remarkably, osteocyte-targeted deletion of LRP5 or overexpression of HBM LRP5 mutants only in osteocytes reproduces the low or HBM, respectively, exhibited by mice or humans with the genetic modifications in all cells, suggesting that activation of the pathway in osteocytes is sufficient to elicit bone formation downstream of LRP5.[68,69] This conclusion is consistent with recent findings demonstrating that osteocytes mediate the anabolic actions of canonical Wnt/βcatenin signaling in bone.[70]

3.3 Regulation of Bone Resorption by Osteocytes and the Role of Osteocyte Apoptosis in Targeted Remodeling

Osteocytes produce cytokines that regulate osteoclast formation and survival. Deletion of the RANKL gene from osteocytes renders an osteopetrotic phenotype, due to reduced number of osteoclasts, decreased resorption, and progressive increase in bone mass,[71,72] suggesting that, postnatally, osteocytes are an important source of RANKL in bone. M-CSF, produced by bone cells including osteocytes,[55] promotes osteoclast precursor proliferation and survival and is required for osteoclast formation.[73] Similar to mice lacking M-CSF in all tissues,[74] mice lacking M-CSF only from osteocytes exhibit reduced osteoclast number and osteopetrosis demonstrating the osteocytes are an important source of the cytokine in bone.[75] Osteocytes also express receptors for M-CSF,[55] and mice lacking osteocytic M-CSF have osteocytes with abnormal morphology, high prevalence

of apoptosis, increased production of reactive oxygen species, and reduced gap junction communication,[75] suggesting that osteocytes are also a target cell for this cytokine.

The antiosteoclastogenic cytokine OPG is expressed in both osteoblasts and osteocytes, although its mRNA expression is higher in the latter cells.[76] OPG is a target gene of canonical Wnt signaling and mice lacking βcatenin from osteoblasts/osteocytes or only from osteocytes, exhibit similar reduced OPG expression, increased osteoclasts, and low bone mass.[76–78] These findings suggest that the regulation of osteocytic OPG by Wnt/βcatenin signaling plays an important role in the control of bone resorption.

Osteocyte viability is an important component of the cascade of events that directs osteoclasts to particular bone surfaces, so-called targeted bone remodeling.[3,79,80] Accumulation of apoptotic osteocytes in certain areas of bone promotes preosteoclast recruitment, local osteoclast differentiation, and increased resorption. Although the mechanisms underlying this phenomenon are still unclear, it is recognized that osteocyte apoptosis precedes temporally and spatially osteoclastic resorption. Induction of osteocyte apoptosis by injecting diphtheria toxin to transgenic mice expressing the diphtheria toxin receptor in osteocytes is sufficient to increase osteoclasts and trigger resorption in the vicinity of dead osteocytes.[81] Moreover, osteocyte death is accompanied by increased osteoclasts in nearby bone in rodent models of unloading, excessive mechanical forces, or ovariectomy, opening the possibility that increased osteocyte apoptosis is a generalized mechanism to induce localized bone resorption.

One of the potential mechanisms by which increased osteocyte apoptosis could trigger local bone resorption is by increasing RANKL expression in osteocytes located close to the dying cells. This notion is supported by the fact that inhibition of apoptosis by administering bisphosphonates that prevent osteocyte apoptosis but do not act directly on osteoclasts or caspase inhibitors blocks the increase in osteocytic RANKL found in the aforementioned animal models.[3,79,80,82,83] Further, deletion of RANKL from osteocytes and mature osteoblasts prevents the decrease in bone mass and the increase in osteoclast number in tail-suspended mice.[71] Moreover, inhibition of osteocyte/osteoblast apoptosis with a bisphosphonate that does not affect osteoclasts or with a pan caspase inhibitor prevented the increase in osteocytic RANKL induced by unloading. However, the bisphosphonate did not prevent the increase in circulating bone resorption markers or the loss of bone,[82] whereas the caspase inhibitor did. In this latter case, however, resorption was evaluated only at the local level in cortical bone, and systemic effects on circulating CTX or bone mass throughout the skeleton were not reported, potentially explaining the apparent discrepancy between the studies. Nevertheless, taken together these findings demonstrate a cause–effect relationship between osteocyte apoptosis and osteocytic RANKL and suggest that, under certain unloading conditions, osteocytic RANKL is central for osteoclast formation and bone resorption.

In addition to osteocytic genes or pathways that regulate either bone formation or bone resorption, genetic manipulations targeting osteocytes can simultaneously control both arms of remodeling. For example, activation of βcatenin in osteocytes increases bone mass in the context of elevated bone formation and bone resorption, leading to high bone remodeling with bone gain.[70] This effect contrasts with the findings that activation of βcatenin in osteoblasts/osteocytes increases bone mass due to reduced bone resorption, without affecting osteoblast function.[78]

4 GENE DELETION AND MUTATIONS AFFECTING OSTEOCYTES

Osteocytes were elevated to their role of master regulators of bone metabolism after genetic studies linked two distinct pathologies, sclerosteosis and van Buchem disease to a protein, sclerostin, made predominantly, if not exclusively, by mature osteocytes. Over the years, the list of osteocyte specific genes linked to disorders of skeletal or mineral metabolism has grown steadily and now it is known that these "unique" cells synthetize hormones, cytokines, and proteins required for phosphate homeostasis and bone integrity. Genome-wide association studies (GWAS) have analyzed hundreds of thousands of single nucleotide polymorphisms and correlate them with skeletal phenotypes. GWAS identified more than 500 genes linked to osteoporosis and 3 predominant pathways that are critical for skeletal biology: Wnt, Notch, and Indian Hedgehog (for review, see Ref. 84). Genes and loci that highly correlate with osteoporosis include, among others, MEPE, Lrp4, Mef2C, HDAC5, all abundantly expressed in osteocytes. Moreover, osteocytes are also important regulators of the Wnt pathway, so we will review genetic disorders affecting bone or mineral metabolism that are primarily related to osteocyte defects.

Canonical Wnt signaling plays an important role in the development and maintenance of many organs, including bone, as described earlier. Several rare genetic disorders affecting the Wnt-signaling pathway have provided strong evidence for its role in skeletal homeostasis. Genetic mutations of various components of the Wnt pathway are associated with skeletal disorders (see following).

4.1 Sclerostin, Sclerosteosis, and van Buchem's Disease

Sclerosteosis was first described by Hansen in 1967 as a skeletal disorder characterized by generalized osteosclerosis with hyperostosis of the calvarium, clavicles, and sternum, and abnormalities of the digits (syndactyly). The disease was distinct from osteopetrosis due to the lack of skeletal fragility or of disturbance of dentition or anemia. In patients with sclerosteosis, the skeletal deformities are not present at birth but they became noticeable in early childhood and progress steadily thereafter. The disease is quite rare and cases have been reported in South Africa,[85] Netherland, Spain,[86] Japan,[87] Brazil,[88] and Egypt.[89] Clinical features of sclerosteosis closely resemble that of van Buchem disease, with the exception of gigantism and hand abnormalities that are not present in the latter. Both diseases were found to be autosomal recessive and they remained of unknown etiology until 1999 when the gene responsible for sclerosteosis and van Buchem disease was localized on chromosome 17g12-q21.[62,90,91] Two years later, changes in sclerostin, the product of the gene SOST, were identified as the cause. Sclerostin, a cysteine rich protein with homology to gremlin, is a potent inhibitor of bone formation and acts by binding and inactivating the canonical Wnt-signaling pathway. Patients with sclerosteosis carry a point mutation in the SOST gene whereas patients with van Buchem disease are characterized by a 52 kb deletion downstream of the gene.[92] Although osteoclasts were initially thought to be the main source of sclerostin[93] soon it became clear that this protein is secreted predominantly, if not exclusively, by osteocytes.[94,95] Sclerostin binds to low-density lipoprotein (LDL)–related protein 5, 6, and 4 (LRP5, 6, and 4) receptors expressed on osteoblasts and osteocytes and inhibits Wnt/βcatenin pathway.[96] Whereas LRP5 and 6 are needed for Wnt-signaling transduction, LRP4 works as an anchor for Sclerostin binding. The pathological role of sclerostin has been further validated in knockout and transgenic animal models that recapitulate the high bone mass phenotype of sclerosteosis and van Buchem patients.[63,97] Recently, craniodiaphyseal dysplasia, a rare and severe bone dysplasia characterized by sclerosis of the skull and facial bones has also been linked to a "de novo" mutation in the SOST gene[61]

4.2 LRP Mutations

The role of canonical Wnt-signaling in bone was first described when loss-of-function mutation of LRP5, a coreceptor for Wnts, were found to cause, in humans, osteoporosis-pseudoglioma syndrome (OPPG), an autosomal recessive disorder characterized by osteopenia and low bone mass.[98] Similarly, heterozygous missense mutation in LRP5 (gain-of-function mutations) leads to the high bone mass phenotype in both humans[69,99] and in mice. Mutation in LRP4 and 6 have also been identified and correlated with genetic disorders. Once again, genetically modified animals have helped to unravel the pathophysiology of LRPs and Wnt/βcatenin mutations and to characterize their underlying mechanisms of action. LRP5-null mice recapitulate the osteoporosis-pseudoglioma phenotype seen in humans, and the low bone mass phenotype in these animals appears to be driven by severely compromised bone formation. Interestingly, when LRP5 is ablated from specific cells of the osteoblastic lineage, the phenotype becomes more complex, suggesting additional mechanisms might be involved. Deletion of LRP5 or βcatenin in osteoblast, using the collagen 1 promoter, or in osteocytes, using the DMP1 promoter, induce marked osteopenia and increased bone resorption.[67,68,78]

The exact underlying genetic mutations and pathophysiology of LRPs and Wnts are discussed in more details elsewhere in this book.

4.3 Mutations Leading to Disturbance of Phosphate Homeostasis

Besides their role on bone mineral metabolism, osteocytes (and possibly late mature osteoblasts) function as an endocrine organ by secreting hormones and factors required for controlling serum phosphate levels. Several phosphate regulating hormones and enzymes, namely, FGF23, PHEX, and MEPE, are synthesized predominantly by osteocytes and, to a lesser extent, by mature osteoblasts. FGF23 was identified in 2000 by White et al. (ADHR Consortium[100]) as the long sought-after phosphate-regulating hormones (phosphatonins) causing autosomal dominant hypophosphatemic rickets (ADHR). Soon after this initial report, FGF23 was shown to cause phosphate wasting in tumor-induced-osteomalacia and X-linked hypophosphatemia (XLH). A few years later, two groups independently demonstrated that autosomal recessive hypophoshatemic rickets (ARHR) was caused by a mutation in dentin matrix protein 1 (DMP1) that affected FGF23 circulating levels.[37,101] Mice lacking DMP1 have skeletal abnormalities (including a disrupted osteocyte-network), are hypophosphatemic and have increased FGF23 levels, faithfully mimicking the mineral and skeletal abnormalities of ARHR patients. Once again the main culprits in this disease are osteocytes. DMP1 is produced by osteocytes and late mature osteoblasts, and it is a negative regulator of FGF23 synthesis and secretion, through unclear mechanisms. Osteocytes are also, together with mature osteoblasts, the main source of FGF23 and recent work demonstrated that targeted ablation of this hormone in bone cells, indeed recapitulate the hyperhosphatemia present in FGF23 null mice.[102] Whereas the role of osteocytes in skeletal metabolisms has been quite well

validated, their role in phosphate homeostasis still has some unanswered questions. When osteocytes are temporarily ablated in mice, using a mouse model in which the diphtheria toxin receptor is targeted to these cells[37] by the DMP1-promoter, the animals, as expected, develop severe osteopenia but their mineral homeostasis remains unchanged. Moreover, FGF23 and phosphate levels are normal, despite the lack of more than 80% of these cells, suggesting the presence of other sources. These findings have puzzled the scientific community and it has been proposed that other cells in the skeleton (maybe endosteal osteoblasts) might be the source of FGF23. Recent work from Clinkenbeard[102,103] used a floxed FGF23 mouse model to demonstrate the role of bone-derived FGF23. By targeting FGF23 deletion in Col2.3- or DMP1-expressing cells the authors demonstrated that both osteoblasts and osteocytes contribute to FGF23 circulating levels and partially explain absence of changes in mineral homeostasis in osteocyte-less mice.

In summary, over the last decade, several genetic mutations and human diseases have been ascribed to osteocyte defects and GWAS has identified osteoporosis "candidate genes" that are highly expressed in these unique cells. Future studies and better understanding of the role of these cells will unravel more functions of the unique bone cells.

5 CROSS TALK OF BONE WITH OTHER ORGANS/TISSUES

Osteocytes are endocrine cells that can secrete factors with the potential to have effects on distant organs. Bone as a mineralized tissue is thought to be more of a support structure and not as an endocrine organ similar to the pituitary or adrenal glands. However, as bone is highly vascularized and secretes factors into the bloodstream to affect distant targets, it must be defined as an endocrine organ.[2] The vascular system has a close, connecting association with the osteocyte lacuna–canalicular system. A bone fluid with a molecular weight cutoff of around 70 kDa flows through this system with the potential to provide autocrine and paracrine factors to neighboring osteocytes and in association with the vascular system, endocrine factors to other organs. As described earlier these osteocytes produce circulating factors, such as FGF23 and sclerostin and, most likely, other as yet unknown factors. Potentially osteoblasts have the capacity to release factors into the circulation, but they compose approximately 5% of bone cells compared to 1% being osteoclasts, whereas 90%–95% of bone cells are osteocytes. Considering the total mass of osteocytes and their dendritic processes in bone that are equivalent to the mass of the brain,[104] these cells are likely a major source of circulating factors from the adult skeleton.

Clearly bone is a source of osteogenic growth factors, such as the bone morphogenetic proteins, the TGF-β, and the insulin-like growth factors. It is thought that these factors are deposited in matrix by osteoblasts and released by osteoclasts during resorption to initiate coupling.[105] One factor produced uniquely by bone is osteocalcin which has been shown to target numerous tissues and is therefore considered a hormone.[106] Osteocalcin is produced by the late osteoblast before embedding into osteoid and also by embedded osteocytes. Like growth factors, it is thought that osteocalcin is mainly deposited into the bone matrix during bone formation and is hypothesized to be released by osteoclasts during resorption.[107] Osteocalcin has high affinity for hydroxyapatite, but with removal of carboxylation potentially by acid, it becomes a circulating factor. Osteocalcin has effects on glucose metabolism, energy metabolism, fertility, ectopic calcification,[108] and on muscle activity.[109] As osteocytes can remove their perilacunar matrix,[22] this matrix is also a potential source of endocrine factors and growth factors.

Osteocytes are mechanosensors and their secreted factors are also regulated by loading or unloading of the skeleton. One major example is the decrease in sclerostin with loading[13] and increase with unloading.[14,110] This response is most likely mediated through the Wnt/βcatenin pathway which serves as an important regulator of bone mass and is important in osteocyte transmission of mechanical loading signals to cells on the bone surface.[76] The pathway is triggered by cross talk with the prostaglandin pathway in response to loading resulting in a decrease in negative regulators of bone formation, such as sclerostin and Dkk1.[11] It has recently been shown that two factors, prostaglandin E_2 and Wnt3a, both produced by osteocytes in response to shear stress support myogenesis and muscle function.[111–114] Therefore, mechanical loading of the skeleton especially in the form of exercise is important to ensure healthy osteocyte function.

5.1 Cross Talk of Bone With Kidney and Heart

Osteocytes regulate mineral homeostasis especially phosphate through factor production, such as phosphate regulating neutral endopeptidase on chromosome X (Phex), dentin matrix protein 1 (Dmp1), and FGF23.[115] Since its identification in 2000, FGF23 has become recognized as one of the most important osteocyte-secreted endocrine factors due to negative effects on kidney and heart.[37,116–118] Under normal physiological conditions, both Dmp1 and Phex downregulate FGF23 in osteocytes allowing reabsorption of phosphate by the kidney to maintain sufficient circulating phosphate to maintain normal bone mineral content. In the absence of either Dmp1 or Phex, FGF23 is elevated in the osteocyte and systemically, leading to phosphate excretion by the

kidney resulting in osteomalacia and rickets.[37] Interestingly, FGF23 is also able to act on the parathyroid gland to decrease PTH secretion, identifying the parathyroid gland as another endocrine target of osteocyte signaling.[119,120]

FGF23 also plays a pathological role in chronic kidney disease (CKD). It was first shown by Pereira and coworkers in 2009 that FGF23 is elevated in osteocytes in CKD.[121] In CKD, serum levels of FGF23 are increased, particularly in the later stages of the disease.[122,123] FGF23 levels predict cardiovascular events before but not after dialysis for CKD and FGF23 is a risk factor for adverse outcomes in patients with CKD and End Stage Renal Disease (for review, see Ref. 124). Therefore, regulation of systemic FGF23 levels has become a significant clinical target.

High circulating levels of FGF23 have negative effects on cardiac muscle (for review, see Ref. 125). Studies have linked elevated levels of circulating FGF23 to increased risk of heart disease and independently associated with left ventricular hypertrophy in human population studies.[126,127] Increased serum FGF23 has also been linked with impaired vascular function,[126] vascular calcification,[128] and increased fat mass.[129] Clearly osteocyte-regulated proteins play an important role not only in normal physiology but also in disease.

5.2 Cross Talk of Bone With Muscle

Bone–muscle cross talk is of interest because of the realization of the implications of this emerging field of research for the diseases of osteoporosis and sarcopenia and potentially others.[125] It is not clear if the two diseases are concurrent or if one precedes the other. With life expectancy increasing, the consequences of aging on the musculoskeletal system will place a greater burden on public health and the economy. It is projected that 20% of the world population will be in their 60s or older by 2050 and over 30% by 2150.[130,131]

Bivariate GWAS has been used to identify pleiotropic candidate genes/single nucleotide polymorphisms/regions associated with traits in both bone and muscle.[132–137] One of the genes identified was *MEF2C*, that encodes a transcription factor (myocyte enhancer factor 2C) known to be involved in cardiac and skeletal muscle development.[138] A mouse model where *Mef2C* was specifically deleted in osteocytes has increased bone density through downregulation of Sost and increased OPG resulting in reduced osteoclastogenesis.[139] Another gene identified was *METTL21C*. Downregulation of this gene in C2C12 muscle cells led to reduced myogenic differentiation, a decrease in myotube cell area, and reduced calcium release from the sarcoplasmic reticulum, while downregulation in MLO-Y4 osteocytic cells resulted in cells more prone to dexamethasone-induced cell death. These effects were linked to the alteration of common signaling pathway, the NF-kB pathway.[140] *METTL21C* is part of the METTL21family of the methyltransferase superfamily with protein-lysine *N*-methyltransferase activity,[141] and methylates valosin-containing protein chaperones. These proteins can harbor specific mutations that are causal for inclusion body myositis (muscle) with early onset of Paget's disease.[142] These studies suggest important roles for MEF2C and METTL21C in both bone and muscle.

Dogma has been the main interaction between muscle and bone in the mechanical loading of bone through muscle contraction. However, not only the bone is an endocrine organ, but also the muscle.[143] Myostatin, is a potent inhibitor of skeletal muscle cell proliferation and growth,[144,145] and in 2007 Pedersen coined the term "myokines," for muscle secreted factors which includes IL-8,[146] brain-derived neutrophic factor,[147] irisin, a potent regulator of the conversion of white fat into brown fat,[148] and IL-15, a muscle factor that reduces adiposity.[149] Secreted factors from C2C12 myotubes but not from C2C12 myoblasts increased the viability of MLO-Y4 osteocyte-like cells treated with dexamethasone[150] and intact skeletal muscles electrically stimulated ex vivo also protect osteocytes against cell death.[150]

Conversely, osteocytes secrete factors that regulate muscle mass and function. MLO-Y4 osteocyte-like cells and primary osteocytes secrete factors that induce muscle myogenesis and activate the Wnt/βcatenin pathway in C2C12 pluripotent cells.[112,113] These findings suggest that this pathway not only plays a role in muscle development but also in muscle repair and myogenesis. Two factors produced by osteocytes in response to shear stress, PGE_2 and Wnt3a, were found to enhance myogenesis and ex vivo primary muscle function. The first in vivo data to suggest that bone regulates muscle mass and function was in 2015 showing that osteocalcin partially restored muscle mass in a model of deletion of Cx43 in osteocytes[151] and these effects of osteocalcin on muscle have been repeated and extended by the Karsenty laboratory in 2016.[109] Also in 2015, Waning and coworkers[152] showed that cancer induced release of TGF-β from bone was responsible for muscle cachexia and Gorski and coworkers[153] showed that deletion of a protease, MBTPS1, in osteocytes resulted in little effects on bone but significant increases in muscle mass and function with aging. Therefore, several lines of in vitro and in vivo evidence are emerging to support the hypothesis that bone can regulate muscle mass and function through secreted factors.

5.3 Cross Talk of Bone and Hematopoietic Cells

Under physiological conditions, the BM is the primary site for hematopoiesis, a process highly orchestrated

by the interactions between the hematopoietic stem cells (HSC) and their niches. Seminal work of two groups,[154,155] identified endosteal osteoblasts as key component of the HSC niche. Over the last decade, the complexity and composition of the HSC niche has been recognized to be much more complicated than expected. Osteoclasts,[156] CXCL12-abundant cells (CAR Cell),[157] perivascular Nestin + cell,[158,159] endothelial and adipose cells[160] have all been shown to be a critical component on the niche (for review, see Ref. 161). Considering the physical proximity and the secretory capability of osteocytes, it is not surprising that these cells also participate in maintaining the HSC niche. Studies from Fulzele et al.[162] showed that osteocytes and GPCR signaling were important in controlling myeloid cells proliferation and mice lacking osteocytes (osteocyte-less animals) were shown to have defective HSC mobilization and lymphopenia.[163,164] Whether osteocytes control the HSC niche directly, through their cytoplasmic extensions, or indirectly, via secreted factors, still awaits further clarification.

5.4 Cross Talk of Bone and Fat

Using a mouse model in which osteocytes can be ablated by use of diphtheria toxin (DT), Sato et al.[164] demonstrated that these cells might also regulate adipose tissue. Upon DT-injections, Osteocyte-less mice had a marked reduction in white adipose tissue including subcutaneous, mesenteric, and retroperitoneal fat. Although in this model the lean phenotype could not be rescued by parabiotic experiments, further studies have demonstrated a role for sclerostin in inducing adipocyte differentiation.[165] Similarly, mice lacking Gs-α signaling in osteocytes and/or osteoblasts also develop a lean phenotype characterized by reduced white adipose tissue and increased brown adipose tissue (Divieti Pajevic et al., unpublished data).

6 THE DYING AND AGING OSTEOCYTE

As described earlier, osteocyte cell death is important to repair damaged bone.[166–168] This cell death acts as a beacon to direct osteoclasts to microdamaged bone for removal as the cells sacrifice themselves to target bone repair. This death is highly programmed to release factors, such as RANKL to direct osteoclasts to damaged sites for repair. Therefore, it is important to maintain the health of the osteocyte. However, it appears that oxidative stress caused by disuse, estrogen deficiency, corticosterone, and aging may be responsible for bone cell death and therefore bone fragility.[169] Osteocyte viability is crucial for the normal functioning of the skeleton and the normal function of other organs, such as kidney and muscle. As osteocytes have multiple functions, outlined earlier, it is important to maintain normal viability and function of these cells.[2] A number of factors and cytokines have been shown to induce osteocyte cell death including glucocorticoids, IL-1 and TNF-α and a number of molecules have been shown to protect osteocytes from cell death, such as estrogen, bisphosphonates (for review, see Refs. 170–172) and PTH. Recently, it has been shown that secreted muscle factors could protect osteocytes from glucocorticoid-induced cell death and that this effect was mediated through the βcatenin pathway.[111] The large number of factors that can induce osteocyte cell death and the large number of protective mechanisms imply the importance of regulating osteocyte cell death and viability.

The osteocyte is an aging, senescent cell. As osteocytes reside in human bone for decades while osteoblasts and osteoclasts live for only days or weeks, the osteocyte is very susceptible to the effects of aging. The osteocyte cannot be replaced except by bone remodeling, therefore the osteocyte should be considered the senescent cell in bone. Osteocyte cell death can result in micropetrosis where mineral fills the lacuna resulting in a cell that becomes a "living fossil."[173] Fewer numbers of osteocyte lacunae were found in patients suffering from fractures compared to controls[174] and an age-dependent decrease occurs in osteocyte lacunar density with an increased amount of hypermineralized calcium phosphate occlusions.[43] Infilling of osteocyte lacunae and canaliculi has important implications for the flow of bone canalicular fluid, that may determine the capacity of the osteocyte to sense mechanical loading and to sense microdamage. The aging osteocyte in a compromised lacuno–canalicular system is less likely to produce secretory factors. Therefore, it is important to maintain osteocyte viability with age.

7 CONCLUSIONS

Whereas the majority of genetic mutations responsible for bone disease are important in embryogenesis, growth, and development, such as collagen mutations, clearly a number of genetic mutations have been found in genes either relatively specific or highly expressed in osteocytes, such as sclerostin, Phex, Dmp1, and FGF23. The number of osteocytes is relatively low in the embryo and increases in number during the growth so that in the mature adult the number of osteocytes outnumber osteoblasts 10–1 and osteoclasts 100–1. When thinking of bone genetic diseases, it will be important to consider these two categories before proposing specific cellular and molecular mechanisms. The osteocyte clearly regulates bone function postnatally and most likely has a progressively greater role in the health of the skeleton with aging.

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CHAPTER

15

Skeletal Stem Cells/Bone Marrow Stromal Cells

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1 HISTORY AND DEFINITIONS OF POSTNATAL STEM CELLS

The notion that postnatal tissues self-renew, and do so by means of a local tissue-specific stem/progenitor cell is not a new one, and is based on observations around the turn of the 19th century by classical histologists (e.g., Regaud working on spermatogenesis[1,2]) and hematologists (e.g., Weidenreich, Dantschakoff, Maximow, Ferrata, and Pappenheim, reviewed in Ref. 3). This concept was originally based on studies of tissues with high proliferative capacity and rate of turnover (blood, gastrointestinal track, epidermis).[4] However, findings since 1990s have demonstrated that virtually all postnatal tissues have some form of a tissue-specific stem/progenitor cell, capable of local regeneration, albeit with different rates of turnover and structural design, depending on the demands imposed upon them (e.g., intestine, mammary gland, and muscle, reviewed in Ref. 5).

1.1 Definitions

While much has been learned through the use of lineage tracing in mice,[6,7] the distinction between a stem cell and a progenitor cell is still somewhat blurry in many cases. However, it is now generally agreed upon that a stem cell is able to: (1) proliferate upon demand to generate progeny that differentiate and reform a tissue (potency), and (2) self-renew (it has the ability to maintain itself as a stem cell in a sea of more committed and differentiated progeny). On the other hand, a progenitor cell, while able to transiently proliferate, is thought to be more committed to a phenotype. Yet, this definition may be somewhat rigid in that there are examples of progenitor cells switching their fate based on changes in the microenvironment (e.g., bone marrow stromal cells, and even committed osteogenic cells being able to form adipocytes indicating their flexibility). However, that is not to say that progenitor cells are able to differentiate into a cell type outside of the lineage of their tissue of origin (so-called "transdifferentiation") without extreme chemical (e.g., treatment with DNA methylation inhibitors) or molecular [e.g., reprogramming with the specific transcription factors to create induced pluripotent stem cells (iPSCs)] modifications. In this context, bone and its stroma represent a unique organ system for the study of stem/progenitor cell kinetics, regulation, and the maintenance of tissue hemostasis.

1.2 Identification of Skeletal Stem/Progenitor Cells

Perhaps the first evidence of the presence of stem/progenitor cells in a connective tissue emanated from the pioneering studies of Alexander Friedenstein in the late 1960s. He demonstrated that when single cell suspensions of murine bone marrow, free of fragments of trabecular bone, were plated at low density, rapidly adherent fibroblastic cells appeared, and proliferated to form a colony composed of bone marrow stromal cells (BMSCs). When individual colonies, established by these colony-forming unit-fibroblasts (CFU-Fs) were transplanted in a closed system, impenetrable by the vasculature (diffusion chamber), a number of them formed bone on the exterior of the chamber (an aerobic environment), and cartilage in the interior (an anaerobic environment). When transplanted in a collagen sponge into an open system that could be vascularized (under the kidney capsule), a number of single colonies were able to reform a bone/marrow organ, composed of bone, hematopoiesis-supportive stroma (a defining feature of these cells) and marrow adipocytes of donor origin, and

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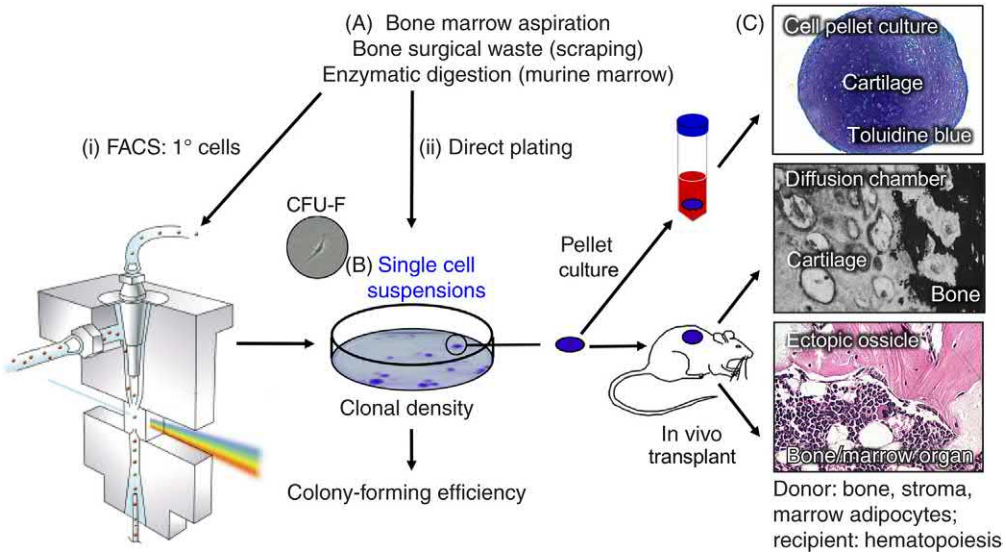

FIGURE 15.1 Isolation and characterization of SSCs/BMSCs. A. SSCs/BMSCs are can be obtained from bone marrow (BM) aspirates or scrapings of marrow from surgical waste, or by enzymatic digestion of marrow, which is used primarily for murine bone marrow. A(i). The freshly isolated single cell suspensions thus generated can be used for FACS sorting based on a variety of cell surface markers, or A(ii), used for direct plating. B. Colony-forming efficiency (CFE) is determined by plating the single cell suspension at clonal density (see text) in order to determine the number of colonies initiated by colony-forming units-fibroblasts *(CFU-Fs)*. This is the closest approximation of the number of SSCs available to date. C. After ex vivo expansion of individual colonies, aliquots are used to generate cell pellet cultures for evidence of chondrogenic differentiation, or transplanted in vivo into immunocompromised mice in conjunction within a diffusion chamber (cartilage and bone), or with an appropriate scaffold. The constructions form an ectopic ossicle (BM/organ), with bone, stroma, marrow adipocytes of donor origin, and hematopoiesis of recipient origin. Support of hematopoiesis is a defining feature of SSCs/BMSCs.

hematopoiesis of recipient origin[8] [Fig. 15.1A(ii)–C]. He, along with his collaborator, Maureen Owen, later called these cells "multipotent bone marrow stromal stem cells," (reviewed in Refs. 9,10). More recently, the term "skeletal stem cell" (SSC) was coined by Paolo Bianco and coworkers based on the ability of individual CFU-Fs to recreate skeletal tissue.[11] He also determined, for the first time, the anatomic location of this elusive cell, and its ability to self-renew,[12] making SSCs bona fide stem cells.

1.3 From SSCs/BMSCs to "Mesenchymal Stem Cells"—A Source for all Mesodermal Derivatives?

The original concept of a tissue-specific skeletal stem/progenitor cell put forward by Friedenstein and Owen was based on rigorous evidence of the inherent properties of BMSCs and its subpopulation of SSCs, and was limited to the skeletal system. As such, these findings were largely unnoticed, except by some, interested in the osteogenic differentiation process (from uncommitted precursor to a fully mature osteocyte), and in harnessing their potential to reconstruct a bone/marrow organ. However, as studies on the bone regenerative properties of SSCs/BMSCs expanded, the original concept was altered. Based on their predominantly mesodermal origin, it was envisioned that SSCs/BMSCs would not only regenerate nonhematopoietic cell types found in the bone/marrow organ, but also other mesodermal derivatives, such as muscle, tendon, ligament, etc. This so-called "mesengenic process" was originally modeled on BMSCs, which were then renamed "mesenchymal stem cells" (MSCs).[13] On the surface, this change in concept was appealing to some, but defied basic principles of developmental biology. "Mesenchyme" is an embryonic connective tissue with broader differentiation properties (able to form not only connective tissues, but also blood and blood vessels).[14] No postnatal stem cell to date (other than iPSCs) has been found to form all three of these types of tissues by rigorous criteria. Furthermore, not even mesodermal derivatives that form bone derive from a common precursor. Somites, derived from paraxial mesoderm, segregate into axial bone-forming tissue (the sclerotome), tendon-forming tissue (syndotome), and muscle- and dermis-forming tissue (dermomyotome); splanchnic lateral plate mesoderm forms bone of the appendicular skeleton[15]; neural crest, derived from ectoderm, forms the facial bones,[16] and lastly, the dorsal root of the aorta gives rise to mesoangioblats that contribute to bone formation.[17,18] During development, there is no common mesodermal (or "mesenchymal") cell for even bone formation, let alone for tendon, muscle, dermis, or other connective tissues.[15]

Although not based in solid science, the thought that "MSCs" derived from bone marrow stroma could

differentiate into mesodermal cell types outside of their normal lineage sparked numerous studies to characterize the biological nature of these cells. Reports emerged suggesting that BMSCs have the ability to differentiation into skeletal muscle, tendon, cardiomyocytes, and so forth, but usually based on extensive chemical modification of culture conditions or genetic engineering, expression of a few markers of the "differentiated" phenotype, and less than rigorous assays to demonstrate their functional capacity (reviewed in Refs. 19–21).

Because of the purported ability to differentiate into cell types outside of their lineage, markers that could effectively isolate multipotent bone marrow (BM)–derived "MSCs" based on their cell surface were intensively investigated. Stro1 emerged as the first such marker, capable of effectively isolating all of the CFU-Fs from BM prospectively.[22] Subsequently, ex vivo–expanded BM-derived "MSCs" were further described as being CD29, CD44, CD71, CD90, CD106, CD120a, and CD124 positive.[23] However, none of these markers are specific for BMSCs, or for the SSC subset within the BMSC population. The majority of these markers is found on many connective tissue cells, and cannot predict a stem cell character (the ability of a single cell to reform a tissue and to self-renew) based on rigorous assays. Furthermore, these markers have most often been used to characterize cells after ex vivo expansion, but it is well known that the pattern of expression of many of these markers change, depending on the culture conditions and length of time in culture, and the value of FACS relates to the use of freshly isolated cells (reviewed in Refs. 20,21) [Fig. 15.1A(i)].

1.4 From Bone Marrow–Derived "MSCs" to Ubiquitous "MSCs"?

Because of the lack of specificity of the reported markers, studies rapidly appeared describing the isolation of "MSCs" from nonskeletal connective tissues. In addition, it became a mainstay to compare the differentiation potential of these nonskeletal "MSCs" to that of BMSCs by using in vitro assays and expression of mRNAs representative of differentiated cell types (as described in Ref. 23), instead of in vivo transplantation (as reported in Refs. 8,24 and by many others). However, current in vitro assays are prone to artifact and misinterpretation, and do not reliably predict the inherent osteogenic, adipogenic, and chondrogenic potential of a cell population (as will be described further, reviewed in Refs. 25,26). These two issues combined to generate a great deal of confusion as to actually what is an "MSC". In an attempt to clarify the nature of these cells, a "recommendation" was made that the name "mesenchymal *stromal* cell" be used (due to the lack of convincing data of stemness in many cases) for cells that are: (1) adherent in culture; (2) express CD105, CD73, and CD90, but lack expression of CD45, CD34, CD14, CD11b, CD79a, and HLA-DR markers; and (3) able to differentiate into osteoblastic, adipogenic, and chondrogenic cells in vitro.[27] However, this new term is also inappropriate for postnatal stem/progenitor cells, which are not embryonic, and are not necessarily stromal in nature (e.g., cord blood "MSCs"). Unfortunately, due to its widespread adoption, this recommendation has contributed greatly to the confusion about the what "MSCs" are, as opposed to highlighting the need to determine the nature of connective tissue–specific stem/progenitor cells, to the extent that it is now commonly thought that "MSCs" are ubiquitous and are equivalent in their differentiation properties,[28] in spite of the lack of rigorous analyses (reviewed in Ref. 20). In essence, making a nonskeletal "MSC" behave like a BMSC is like trying to stick the proverbial square peg into a round hole, and the overemphasis on artificial assays overshadows efforts to reveal the inherent differentiation potential of nonskeletal connective tissue stem/progenitor cells.

1.5 "MSCs" are Pluripotent?

Along with the explosion in studies aimed to characterize the nature of BMSCs, and subsequently nonskeletal "MSCs," the thought emerged that BMSCs and "MSCs" are pluripotent. This new twist was based on early studies that aimed to treat generalized disorders of the skeleton, and other mesodermal tissues by direct orthotopic injection or systemic infusion of BMSCs. What followed were suggestions that BMSCs were able to "transdifferentiate" into cell types outside of the mesodermal lineage, such as neuronal cell types, hepatocytes, insulin-producing cells, and so forth [reviewed in Ref. 19). The vast majority of these studies and others related to nonskeletal "MSCs" relied on the expression of a few markers, and did not convincingly demonstrate functionality of these "transdifferentiated" cells. In some cases, formation of a differentiated cell of donor origin could be attributed to cell fusion, which is a natural process in some tissues.[29,30] Based on more recent studies, it is now apparent that "transdifferentiation" of SSCs/BMSCs is a rare event, if it occurs at all (reviewed in Ref. 20). Yet a read of current literature indicates that this concept is still widely held by many scientists; for not only BMSCs, but also for nonskeletal "MSCs." However, the lack of transdifferentiation does not mean that tissue-specific stem/progenitor cell populations are not useful in molecular reprogramming into iPSCs using current methods (transduction with specific transcription factors, modification with mRNAs or small molecules).[31] Rather, it is important to highlight that reprogramming is not equivalent to "transdifferentiation" as originally reported.

2 ORIGINS OF SKELETAL STEM CELLS/ BONE MARROW STROMAL CELLS

The identity of local stem/progenitors has long been sought, but the lack of specific markers of a stem/progenitor cell in any connective tissue has made this a challenging task. However, recent studies suggest that pericytes, cells located on the extravascular surface of blood vessels, may be the source of tissue-specific stem/progenitors that emerge through what might be a common developmental process in many connective tissues.[12,15,20]

During development of BM, blood vessels gain access to what will become the medullary cavity by invading through a layer of committed osteogenic cells.[32,33] Due to an association of the osteogenic cells with endothelial cells, they change not only their morphology, but also cease to synthesize and deposit bone matrix, and proliferate along with the developing blood vessel.[33,34] These former osteogenic cells, now pericytic cells, further proliferate to establish the BM stroma upon which hematopoiesis occurs[33,34] (Fig. 15.2B). In this regard, CD146 (Muc18/MCAM) was demonstrated to mark pericytes on BM sinusoids, and to be a useful marker in isolating pericytes from human BM,[12] providing the first evidence that pericytes are CFU-Fs (Fig. 15.3A–B). Prospective cell sorting for $CD45^-/CD34^-/CD146^+$ cells isolated all of the CFU-Fs in human BM, and after clonal expansion and in vivo transplantation, ~10%–20% of these prospectively isolated CFU-Fs were determined to be multipotent: able to form bone, hematopoiesis-supporting stroma, marrow adipocytes and pericytes of donor origin upon in vivo transplantation (Fig. 15.3B). Of note, these cells were positive for all of the other markers that have been associated with "MSCs," but making them tissue-specific stem/progenitor cells of a different type: $CD146^+$ pericytes. Confirmation of the osteogenic origin of BM sinusoidal pericytes has been reported by lineage tracing using the bone-specific transcription factor, Osterix.[35]

Studies of murine BM using nestin,[36] Mx1,[37] and leptin receptor[38] have also eluded to the pericytic nature of skeletal stem/progenitor cells. Interestingly, pericytes have been identified in other connective tissues as the source of local/stem progenitor cells, such as in dental pulp and in adipose tissue based on Stro1 and CD146 expression, respectively[39,40] and in muscle based on alkaline phosphatase expression.[41] But then,

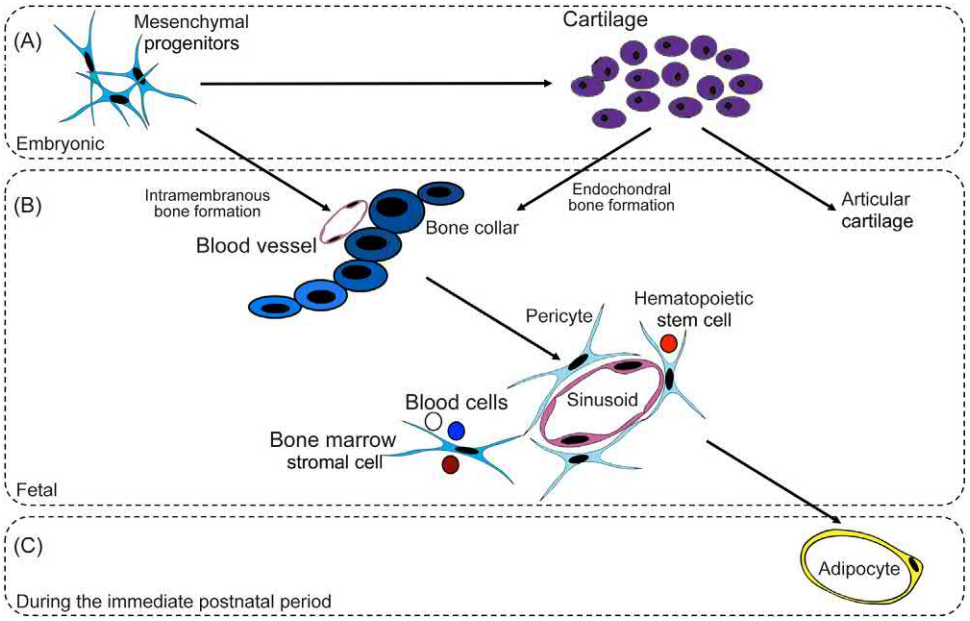

FIGURE 15.2 **Prenatal development of bone and its marrow.** A. At early stages of development, embryonic mesenchyme condenses and forms provisionary cartilage. B. As fetal development continues, the vast majority of the cartilage anlagen undergoes hypertrophy and is replaced by bone, while it remains as articular cartilage in certain sites. In long bones, bone formation begins with the formation of the bone collar by an intramembranous process. Blood vessels associate with committed osteogenic cells in the bone collar and incorporate them as pericytes on the abluminal wall of sinusoids as vascular invasion of the hypertrophic cartilage proceeds. These pericytes further elaborate the prehematopoietic bone marrow stroma. Subsequently, hematopoietic stem cells migrate from the fetal thymus, liver, and spleen to establish hematopoiesis in the newly formed medullary cavity. C. After birth, when hematopoiesis has been established at the level necessary to sustain the organism, bone marrow stromal cells transition into marrow adipocytes. *Source: Modified from Bianco P, Robey PG. Skeletal stem cells.* Development *2015;***142***(6):1023–7.*[15]

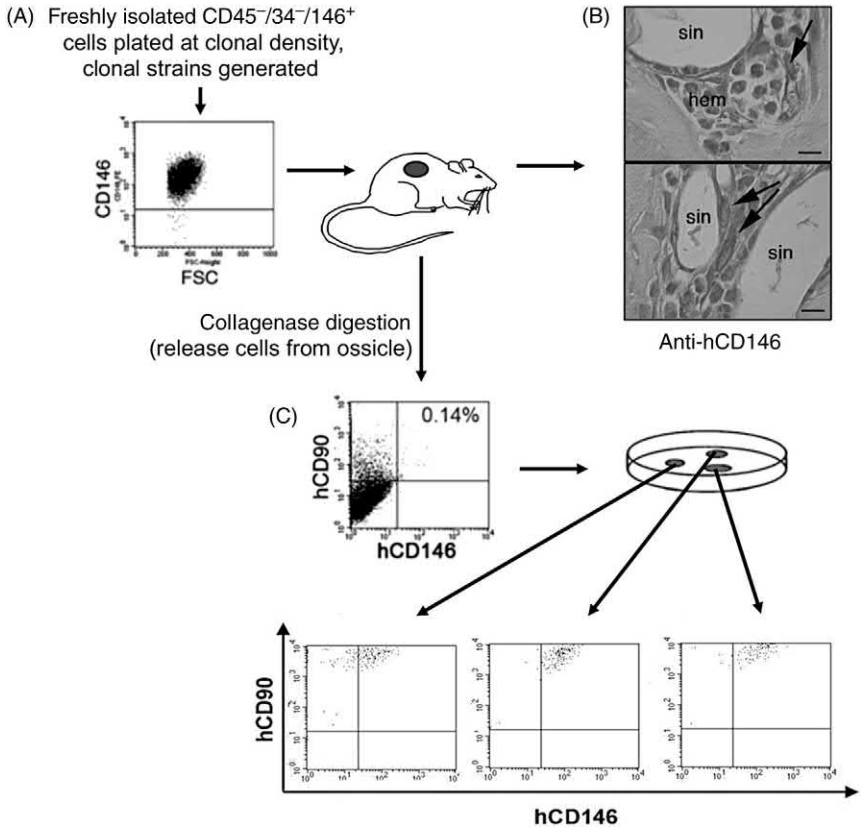

FIGURE 15.3 Identification of SSCs as self-renewing CD146⁺ pericytes. A. In searching for a cell surface marker that would enrich for SSCs, CD146 emerged as an appropriate candidate. Ex vivo–expanded CD146⁺ cells were used to generate in vivo transplants. B. Histological analysis revealed that these cells formed a complete bone/marrow organ, but in addition, reformed CD146⁺ pericytes on BM sinusoids. C. After collagenase digestion of the ectopic bone/marrow organ to liberate cells, human CD146⁺ cells (also hCD90⁺) were isolated and found to be clonogenic, and maintained CD146 expression, indicative of their ability to self-renew. *Source: Modified from Sacchetti B, Funari A, Michienzi S, et al. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment.* Cell *2007;***131***:324–36.*[12]

another twist, based on the fact that "MSCs" share a common cell surface phenotype, and that some "MSCs" are also CD146⁺ pericytes, it was reported that pericytes from all tissues are the true "MSCs." However, this assertion, that pericytes from all tissues are osteogenic, adipogenic, and chondrogenic, was overly dependent on in vitro differentiation assays.[42] This study suffered from all of the issues noted previously with regards to the reliability of in vitro differentiation assays and expression of markers, and did not provide solid evidence to support the claims. However, a recent study using CD45⁻/CD34⁻/CD146⁺ cells from BM, muscle, and umbilical cord blood have shown that they have the ability to form pericytes using an in vivo angiopoiesis assay,[43] providing proof of principle. More tissue-specific stem/progenitor cells need to be analyzed in this fashion to determine if this is really a shared paradigm operating in different connective tissues. However, it must be noted that the developmental origin of pericytes varies from tissue to tissue (they are not a lineage, just as "MSCs" are not a lineage), and their biology is complex and not well understood to date (reviewed in Refs. 44–46). Nonetheless, it can be envisioned that as nascent blood vessels (which are pericyte-free) grow into a developing tissue composed of cells that are at early stages of commitment to a particular lineage, endothelial cells capture the local cells with certain cell surface characteristic to form pericytes, thereby giving the blood vessel stability.[47] As tissue and blood vessel development are completed, these hijacked cells remain quiescent until they are liberated from the blood vessel wall during tissue turnover, or due to injury, at which point they reveal their inherent differentiation capacity[20,43] (e.g., SSCs, Fig. 15.4). In support of the thought that pericytes are committed to a particular lineage, it can be noted that transcriptome analysis of "MSCs" isolated from different tissues do in fact display their inherent differentiation preference; for example, undifferentiated SSCs/BMSCs express low levels of osteogenic, chondrogenic, and adipogenic transcription factors and matrix proteins,[12] while muscle-derived cells express myogenic transcription factors.[43]

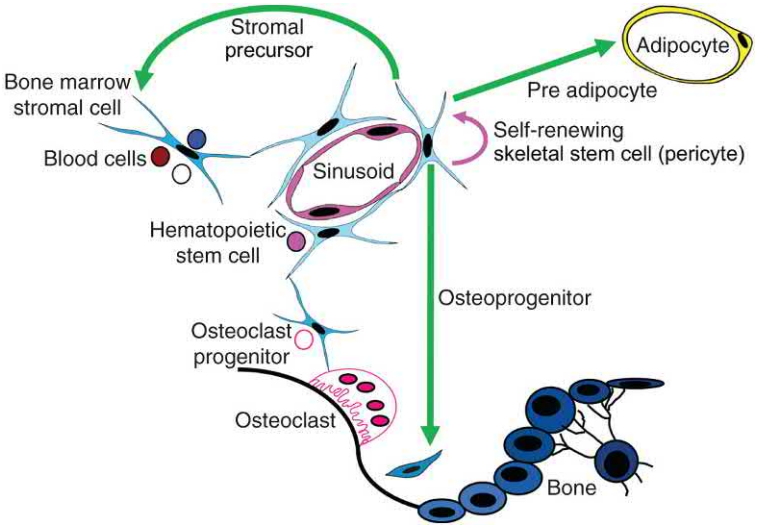

FIGURE 15.4 The role of SSCs/BMSCs in skeletal homeostasis (bone turnover and remodeling). By virtue of their role in hematopoiesis, SSCs/BMSCs support the formation of osteoclastic progenitors, and based on their expression of RANKL, they play a role in osteoclast formation. The pericytic SSC, residing on the abluminal side of marrow sinusoids is quiescent until activated by the need for bone turnover or extrinsic changes in the BM microenvironment, at which point, it is driven to an osteogenic, adipogenic, or stromal cell fate [*dark gray* (*green* in the web version) *arrows*].

3 PRACTICAL CONSIDERATIONS—PREPARATION AND ASSESSMENT OF SSCs/BMSCs

3.1 Isolation

Many different methods have been published for the isolation of SSCs/BMSCs, but generally two techniques are utilized: (1) BM aspiration or lavage of bone fragments containing marrow and direct plating, and (2) FACS sorting of freshly isolated BM using a combination of negative and positive markers (e.g., for human, $CD45^-/CD34^-/CD146^+$ cells, other sorting strategies have been used as well) (reviewed in Refs. 25,26) [Fig. 15.1A(i–ii)]. The volume of an aspirate should be small (~2–5 mL) with several repositionings of the aspiration needle in order to avoid dilution of the marrow with peripheral blood, which can have a negative impact on growth of SSCs/BMSCs.[48] When using human BM, isolation of BMSCs generally does not require the use of enzymatic digestion; however, enzymatic digestion has been found to be necessary for optimal isolation of murine BMSCs.[49,50]

3.2 Culture Conditions

Both murine and human BMSCs are extremely sensitive to culture conditions, and the quality of ex vivo–expanded cells depends on optimization of culture conditions. In particular, fetal bovine serum (FBS) must be lot-selected and used at a level of 20%.[24] Furthermore, it has been shown that heat-inactivation has a negative effect on BMSC growth.[51] In addition to the medium composition, atmospheric conditions also impact cell growth. It has been suggested that hypoxia (2% O_2) is more effective in maintaining stem cells within the SSC/BMSC population than atmospheric conditions (20% O_2).[52] While it is clear that low oxygen increases the rate of proliferation, preliminary results of in vivo transplantation indicated that cells grown in low oxygen do not seem to support hematopoiesis to the same extent as cells grown in atmospheric oxygen (Balakumaran and Robey, unpublished results). More in vivo testing is necessary. Furthermore, addition of certain growth factors can alter the potency of the SSC/BMSC population. For example, bFGF has been demonstrated to increase BMSC proliferation; however, upon in vivo transplantation, the cells made copious amounts of bone, but failed to support the formation of marrow, which is where the SSC resides.[12] This indicates that bFGF treatment does not support self-renewal of SSCs and directs them into an osteogenic fate[12] (or increases chondrogenesis[53]). It also should be noted that optimal ex vivo expansion conditions for murine BMSCs are very different compared with human BMSCs,[54] a fact that is often not recognized.

3.3 Characterization of the Biological Nature of SSCs/BMSCs

Characterization of the SSC/BMSC population after ex vivo expansion is a critical step not only for using them in in vitro experiments, but also for devising processes for their use in tissue engineering (TE) (as described later). Scaling up to generate the numbers of cells

needed for clinical applications do not always recapitulate what is done in the laboratory, or generate populations of cells with equivalent biological activities.

3.3.1 Cell Surface Markers

Cell sorting is essential to define the character of freshly isolated cells, but FACS does not yield sufficient numbers of cells for use in extensive ex vivo experiments, and for bone regeneration studies and clinical applications, so the cells must be expanded in culture. Although the cell surface markers used are not specific, assessment of the cell surface profile after cell expansion does provide some important information on the nature of the cell population. Due to the fact that cell surface markers can change in vitro, loss of certain markers that play important roles can be indicative of less than optimal culture conditions, and overexpansion and senescence. In addition to being negative for hematopoietic markers, they are also negative for endothelial markers [CD31 (PECAM-1), CD62E (E-Selectin)]. In humans, SSCs/BMSCs cells are generally positive for CD13 (aminopeptidase N), CD29 (β1 integrin subunit), CD44 (hyaluronan receptor), CD49α (α1 integrin subunit, VLA), CD63 (lysosomal membrane-associated glycoprotein 3, LAMP3), CD 73 (ecto-5′-nucleotidase), CD90 (Thy-1), CD105 (endoglin), and CD106 (VCAM-1, α4β1 integrin ligand) (reviewed in Ref. 55). Some of these markers are involved in cell–matrix interactions (CD29, CD44, CD49a), while others may be critically important in the biological function of SSCs/BMSCs, such as CD105 (endoglin), which is the regulatory subunit of the TGF-β receptor that modulates responses to TGF-β. Furthermore, based on the potential pericytic nature of SSCs/BMSCs, it is also important to assess the expression of CD106 (Vcam1, murine cells[50]) and CD146 in human cells,[12] which are suggested to regulate interactions with endothelial cells. Other pericyte markers of note are NG2, a cell surface chondroitin sulfate proteoglycan that is expressed by pericytes.[56]

3.3.2 Colony-Forming Efficiency

To date, the colony-forming efficiency (CFE) assay is the closest approximation of the number of SSCs present in freshly prepared single cell suspensions of BM (Fig. 15.1B). However, it must be kept in mind that while all SSCs are CFU-Fs, not all CFU-Fs are SSCs, based upon in vivo transplants of single colony-derived strains. Currently, simple plastic adherence is the most effective way to separate human SSCs/BMSCs from hematopoietic cells, although this is not the case with murine SSCs/BMSCs, which are heavily contaminated with macrophages. In this assay, single cell suspensions are plated at low density ($0.14–14.0 \times 10^3$ nucleated cells/cm^2 for BM aspirates, $0.007–3.5 \times 10^3$ nucleated cells/cm^2 core biopsies). The most accurate determination of CFE is obtained using bone fragments with marrow, based on variable dilution of BM aspirates with peripheral blood.[26,55] When this assay is performed using freshly sorted cells to eliminate hematopoietic cells (e.g., $CD45^-/CD34^-/CD146^+$ cells), clonal density is 1.63–163.0 cells/cm^2. Single CFU-Fs rapidly adhere (2–3 h), and within 24–48 h, begin proliferating to form colonies, demonstrating density-independent growth.[57] Interestingly, to date, sorting with different combinations of cell surface markers and applying appropriate calculations to account for removal of hematopoietic cells results in a similar CFE as that obtained by simple plastic adherence.[12] CFE for human SSCs/BMSCs ranges from 10 to 50 per 10^5 nucleated cells, and does not change dramatically with aging,[58] (Fig. 15.5B, left panel), although the number of population doublings is reduced in BMSCs from aged compared with young donors.[59,60] Substantial differences in CFE can signify either inadequate culture conditions, or a potential skeletal pathology[61] (Fig. 15.5B, middle and left panels). Secondary CFE can be interrogated after ex vivo expansion, but it must be noted that in the secondary assay, transiently amplifying cells (cells that are more committed or differentiated than SSCs, but still proliferative) are also enumerated. CFE may appear to increase, but that does not signify an increase in the number of SSCs without assessment of potency by in vivo transplantation. On the other hand, a significant decrease or loss of colonies would again signify an inefficient ex vivo expansion or pathology.

3.3.3 Demonstration of Bona Fide Stem Cell Characteristics

Differentiation assays are most often performed on BMSC populations as a whole, and as such, are useful in ensuring that SSCs within the population have not been lost during ex vivo expansion. However, clonal analysis and subsequent assays that rigorously demonstrate differentiation potential and self-renewal are essential to support the claims that SSCs reside within the population (Fig. 15.3A–C).

3.3.3.1 Differentiation Potential—In Vitro

Unfortunately, in vitro assays that are widely used to demonstrate osteogenic and adipogenic differentiation potential are highly prone to artifact, and have become (poor) substitutes for in vivo transplantation. Treatment of cells at high density with high levels of β-glycerolphosphate often induces dystrophic calcification formed by dying and dead cells rather than matrix mineralization,[63] yet both forms of mineralization stain positively with Alizarin Red S and von Kossa. In addition, treatment of cells with BMPs is not an appropriate assessment of inherent osteogenic capacity, due to the fact that BMPs will transiently convert virtually any fibroblastic cell into an osteoblast-like cell type as has long been known from the pioneering work of Marshall Urist.[64]

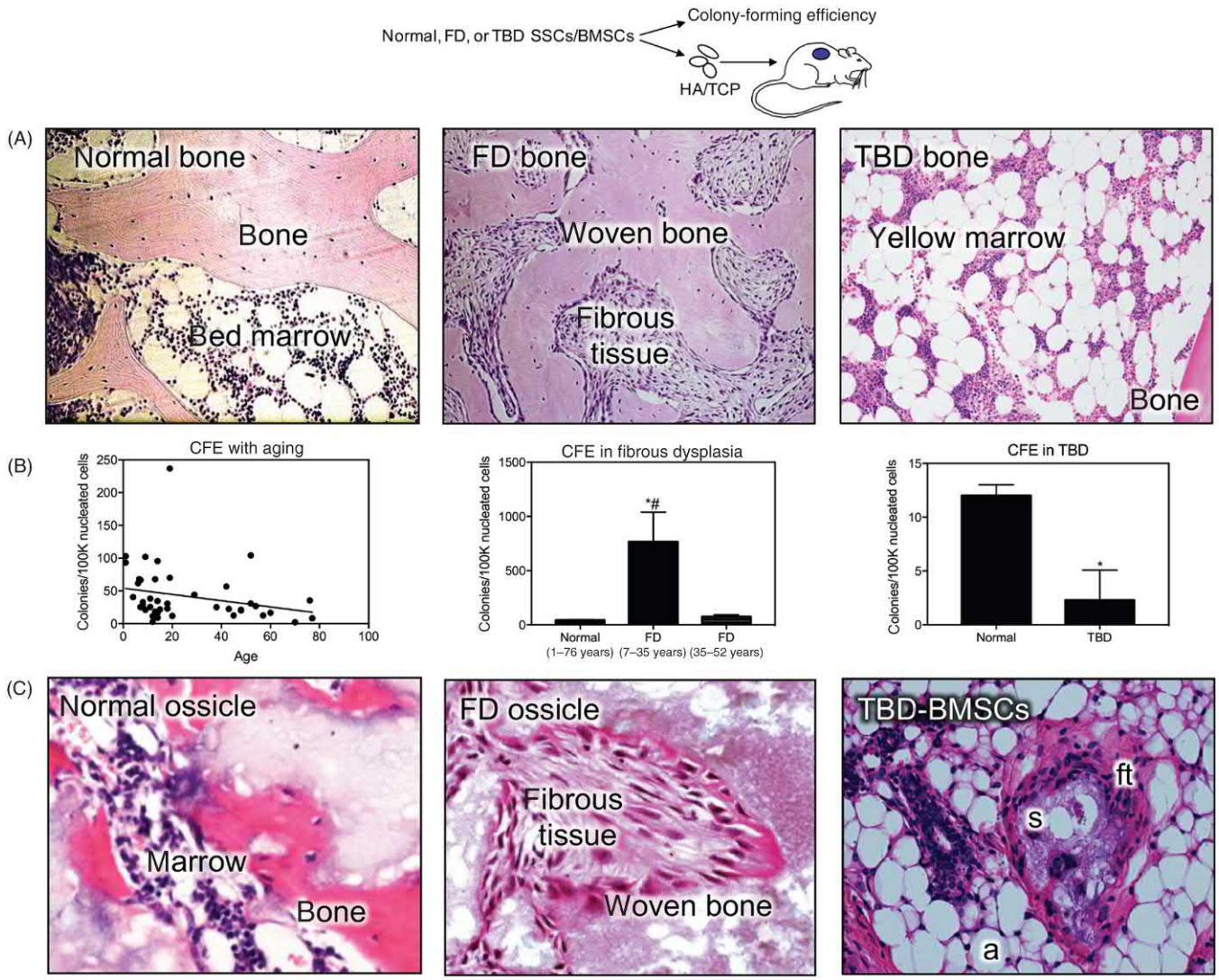

FIGURE 15.5 The role of SSCs/BMSCs in skeletal and hematological diseases. Normal and pathological specimens of BM are used to determine the CFE, the closest approximation of the number of SSCs currently available, and to create ectopic bone/marrow organs upon transplantation into immunocompromised mice. A. Compared to normal marrow (left panel), marrow from patients with the somatic mosaic disease, fibrous dysplasia of bone (FD), is characterized by poorly mineralized woven bone, and replacement of marrow with a fibrotic tissue devoid of hematopoiesis and marrow adipocytes (middle panel). In patients with a telomere biology disease (TBD), hematopoietic red marrow is replaced with yellow marrow (right panel). B. CFE of normal marrow is ~50 colonies/10^{-5} marrow nucleated cells. While there is a gradual decrease with aging, the change is not statistically significant (left panel). In FD, there is a marked increase in CFE in patients under the age of 35 years, but CFE normalizes with age ($P > 0.05$, * in comparison with normal CFE, # in comparison with CFE from patients over 35 years in age) (middle panel). CFE was dramatically decreased in patients with TBD ($P > 0.05$, * in comparison with normal CFE) (right panel). C. Transplantation of normal SSCs/BMSCs reformed a complete bone/marrow organ (left panel). FD SSCs/BMSCs from young patients recapitulated the formation of an FD lesion (middle panel), whereas cells from old patients formed a normal bone/marrow organ (data not shown, presented in Ref. 62). Taken together with the CFE data from young and old patients, it was surmised that the mutated SSCs did not self-renew, whereas the nonmutated SSCs were able to survive and reform a BM organ.[62] SSCs/BMSCs from patients with TBD established a transplant devoid of hematopoiesis, and filled with yellow marrow (right panel).

For adipogenic assays, cells sometimes accumulate lipids from serum rather than synthesize them de novo.[65]

Unlike for osteogenesis and adipogenesis, for chondrogenesis, the gold standard is the in vitro cell pellet culture, although cartilage is also formed in diffusion chambers along with bone (Fig. 15.1C). In the cartilage pellet, one can histologically analyze the tissue formed, and can see bona fide chondrocytes lying in lacuna, surrounded by matrix that stains purple with toluidine blue (metachromasia).[66] The metachromatic staining by toluidine is more specific than staining with alcian blue (which will also lightly stain osteoid), or staining with safranin O (which is also a nuclear stain in addition to a cartilage matrix stain). In many cases, faint staining of micromass cultures or pellet cultures with alcian blue or safranin O has not convincingly demonstrated chondrogenic

differentiation. Of note, chondrogenic differentiation of SSCs/BMSCs is not enduring. Pellet cultures undergo hypertrophy in vitro[67] and in vivo,[68] or remodel directly into a BM organ in vivo.[69] The fact that postnatal SSCs/BMSCs have chondrogenic potential may lie in the fact that before vascularization of hypertrophic cartilage in a developing bone rudiment, some "borderline" chondrocytes do not undergo apoptosis, and become osteoblastic cells.[70] These cells can just as easily be captured by invading blood vessels (like committed osteogenic cells) to form a pericyte, but with a distant memory of its previous life as a chondrocyte.

It is the overutilization and/or misinterpretation of these in vitro assays that has led to the flawed notion, that virtually any fibroblastic population ("MSCs" from nonskeletal tissues) has the inherent capability to form bone, fat, and cartilage. There are very few studies that have performed in vivo transplantation assays on cells derived from nonskeletal connective tissues (without treatment with BMPs or molecularly engineered) to show that mineralized matrix (rather than dystrophic calcification) is formed, with osteocytes, osteoblasts, hematopoiesis-supportive stroma, and marrow adipocytes of donor origin. Furthermore, mRNA expression of a few markers is often used as evidence for osteogenic, adipogenic, and chondrogenic phenotype. However, in most cases their level of expression is not compared to a positive control in order to show the magnitude of expression, and the pattern of expression often does not match the pattern exhibited by SSCs/BMSCs during differentiation. Consequently, expression of markers is not sufficient in the absence of showing differentiation into a functional tissue (reviewed in Refs. 26,71).

3.3.3.2 Differentiation Potential—In Vivo

Based on in vivo transplantation with an appropriate scaffold,[24] there is substantial evidence by using single colony-derived strains that a subset of cells within the BMSC population are multipotent (Fig. 15.1B–C). In this regard, the choice of a scaffold for in vivo analyses must be carefully evaluated. Generally speaking, murine BMSCs are able to recreate a bone/marrow organ in both collagen sponges and high-quality ceramic particles, whereas human BMSCs show a strong preference for ceramics. Of note, most commercially available ceramics are sold as "bone fillers" and are not necessarily osteoconductive.[25] In vivo transplantation assays have shown that some of them support a limited amount of bone formation, but do not support the formation of a hematopoietic marrow, and some do not even support bone formation (Kuznetsov and Robey, unpublished results). Using optimized culture conditions and scaffolds, ~10%–20% of the single cell-derived strains were found to recreate a bone/marrow organ.[8,72–74] This highlights the fact that SSCs are a fraction of the population, and that not all BMSCs are multipotent. Thus, one does not establish a culture of stem cells, as is often stated in the literature; one establishes a culture in which a subset of cells is comprised of stem cells. That is not to say that BMSC populations as a whole are less useful than single colony-derived strains, it is only to say that it is necessary to document the existence of multipotent (or unipotent) stem cells within the population via clonal analysis and appropriate differentiation assays, which primarily rely on in vivo transplantation, although in some cases, in vitro assays are required (cartilage pellet culture).

3.3.3.3 Self-Renewal

The ability to proliferate extensively, in vitro has often been used as evidence of self-renewal. However, extensive proliferation does not indicate that the ex vivo–expanded SSCs/BMSCs (or any other "MSC" population) have maintained: (1) their differentiation capacity or (2) their ability to self-renew (maintained a subset of SSCs). Assays of SSC self-renewal are challenging due to the solid, rather than fluid, nature of bone, and its marrow. The serial repopulation assay of the hematopoietic system is the most rigorous assay of self-renewal available, which serves as a model for developing assays of self-renewal in other tissues. Based on this model, it was reported that SSCs do indeed self-renew.[12] In this study, prospectively isolated human $CD146^+$ cells were used to generate clones that were then transplanted in vivo. Human $CD146^+$ cells were again identified on the surface of sinusoids, and human cells were isolated after enzymatic release from the transplants and found to be $CD146^+$. When plated at clonal density, individual clones were isolated and again found to be $CD146^+$. This study clearly demonstrated that the progeny of a single CFU-F with the same cell surface phenotype as the original explanted cell could be retrieved from the transplant, indicative of self-renewal[12] (Fig. 15.3).

4 THE ROLE OF SSCs/BMSCs IN POSTNATAL BONE TURNOVER

Bone turnover is initiated in response to release of parathyroid hormone (PTH) from the parathyroid gland when the calcium-sensing receptor senses a decrease in serum calcium, and when there is a need to replace bone that has become microdamaged. Formation of osteoclasts from monocyte precursors bearing RANK occurs in the vicinity of the bone surface that is destined to be remodeled. The formation of osteoclast precursors in the monocyte/macrophage series (Fig. 15.4), as well as T cells that impact on osteoclast formation,[75] are supported by SSCs/BMSCs by providing a "bed" [στρώμα (Greek)—*strṓma*] upon which they are formed. SSCs/BMSCs express a long list of hematopoiesis-associated

cytokines and growth factors [Gene Ontology categories (GEO GSE64789) "hematological system development and function" and "hematopoiesis" are highly over-represented in multipotent SSC/BMSC clonal lines[74]]. SSCs/BMSCs also express high levels of M-CSF and RANKL, both of which are essential for osteoclast formation, and osteoprotegerin (OPG), which serves as a decoy receptor for RANKL preventing osteoclast formation. Osteoclast formation and bone resorption is dependent on the balance of RANKL and OPG expressed by BMSCs and more mature osteoblastic cells, and BMSCs have long been thought to play a major role in osteoclast formation.[76] Recently, based on mouse models with conditional or cell/tissue-specific deletion of RANKL, it has been proposed that osteocytes also control osteoclast formation based on their expression of RANKL. However, it appears that the RANKL$^+$ cell type that controls osteoclastogenesis may be site specific. For example, mice with RANKL deletion in mature osteoblasts and osteocytes using a DMP1-Cre driver maintain tooth eruption, which requires bone resorption to occur, whereas the long bones exhibit an osteopetrotic-like phenotype (reviewed in Refs. 77,78). Further studies are needed to determine the contribution of SSCs/BMSCs, more mature osteoblastic cells and osteocytes to initiating osteoclast formation throughout the skeleton.

Subsequent to the formation of osteoclasts and dissolution of mineralized matrix, a plethora of growth factors, buried within bone due to their affinity for carbonate-rich apatite, are liberated, including BMPs, PDGF, IGF-I and IGF-II, and TGFβs. Consequently, PTH, the released factors, as well as those synthesized by local cells (Wnt10b by CD8$^+$ cells, FGF and VEGF by BMSCs), mediate the reversal stage, which is marked by a cessation in bone resorption and creation of a microenvironment conducive for bone formation, thereby coupling bone resorption with bone formation (reviewed in Refs. 78,79). It is currently thought that through osteoclastic action, active TGFβ is released and establishes a gradient between the resorption sites through the marrow to the pericytes located on a nearby blood vessel. This gradient has two effects. Due to the high concentration of TGFβ at the site of resorption, it inhibits migration of osteoclastic precursors into the area; however, the gradient initiates migration of SSCs/BMSCs into the area in need of restoration (Fig. 15.4), but does not induce differentiation.[80] Upon arrival in the resorption bay, BMSCs are induced to osteogenic differentiation by the interplay of the TGFβ/BMP, Wnt, and IGF-I signaling pathways. Interestingly, PTH, in addition to initiating bone resorption, also plays a role in bone formation. PTH bound to its receptor, PTHR1, complexes with Lrp6 (Wnt coreceptor), which leads to the stabilization of β-catenin and pSmad1 signaling, along with stimulating cAMP-mediated signaling. The PTH–PTHR1 complex can also bind to the TGFβRII receptor, leading to pSmad2/3 signaling (reviewed in Ref. 79). IGF-1 signaling activates mTOR via the PI3K-Akt pathway and induces osteogenic differentiation of BMSCs. Alterations in any of these pathways due to mutation, or alterations in levels of expression due to changes in the microenvironment, can have significant consequences on the balance of bone resorption and formation necessary for skeletal homeostasis (reviewed in Refs. 78,79).

5 THE ROLE OF SSCs/BMSCs IN DISEASE

SSCs/BMSCs are central mediators of skeletal homeostasis not only because of their role in bone formation, but also in control of bone resorption due to their expression of RANKL, which controls the formation of osteoclasts through interactions with RANK on osteoclastic precursors. Thus, it was hypothesized that any intrinsic change (mutation) would result in a skeletal disease or disorder. Proof of principle of this concept came from studies of fibrous dysplasia of bone (FD) and the McCune–Albright syndrome (MAS). FD/MAS is a somatic-mosaic disease caused by activating missense mutations of *GNAS*, which encodes for Gsα, leading to overproduction of cAMP. The impact of this mutation on the activity of the SSC/BMSC population (which express Gsα) is profound, leading to the formation of osteomalacic woven bone and replacement of marrow with a fibrotic stroma[81–83] (Fig. 15.5A, middle panel). Although CFE appeared to increase in this disease (Fig. 15.5B, middle panel), the mutated cells were found to be transiently amplifying BMSCs, and were devoid of SSCs.[62] When the cells were transplanted in vivo into immunocompromised mice, they recapitulated the formation of an abnormal bone and fibrotic tissue (devoid of marrow), completely reminiscent of native FD lesions[84] (Fig. 15.5C, middle panel). These studies demonstrated that independent activation of Gsα leads to extensive proliferation of SSCs/BMSCs (transiently amplifying cells), some of which differentiate into misfunctioning osteoblasts that are not able to mineralize osteoid due to their overproduction of the phosphate-regulating hormone, FGF23.[85] Furthermore, it was shown that the mutation prevented self-renewal of SSCs based on the disappearance of mutated SSCs from FD lesions as a function of aging.[62]

Based on their known ability to support hematopoiesis, and the presence of the SSC in the hematopoietic stem cell niche,[86] it was hypothesized that SSCs/BMSCs may also play a role in hematological disorders. One study utilized SSCs/BMSCs from patients with an inherited form of aplastic anemia (Fig. 15.5A, right panel) caused by mutations in genes controlling telomere length [telomere biology disorders (TBD)], such as *TERT*, *TERC*, and a number of other genes (reviewed in

Ref. 87). Dyskeratosis congenital is an example of a TBD characterized by oral leukoplakia, nail dystrophy, abnormal skin pigmentation, high rates of BM failure, growth retardation, and osteopenia. It was determined by performing the CFE assay that the number of SSCs from patients with a TBD was dramatically reduced compared to normal controls (Fig. 15.5B, right panel), and that upon in vivo transplantation into immunocompromised mice, they failed to form bone, but instead formed extensive areas of yellow marrow, devoid of hematopoiesis (Fig. 15.5C, right panel), a replica of misfunctioning marrow in these patients.[88] While it is also known that hematopoietic stem cells are abnormal in TBD, this study suggests that SSCs/BMSCs are a potential therapeutic target for certain types of hematological diseases and disorders.

These two studies provide examples of the power of in vivo transplantation of mutated human SSCs/BMSCs into immunocompromised mice; that is, the ability to make a piece of abnormal human bone and marrow in a small animal model. This model is amenable to the development of new therapies for treatment of skeletal diseases, either by treatment with factors or small molecules to reverse the effects of the mutation, or by the development of gene correction methodologies either in vitro for generation of corrected cells that could be used in TE, or even of gene correction in vivo,[89] based on rapidly evolving gene editing methods, such as CRISPR/Cas9.[90]

Along the same vein, iPSCs derived from patients with genetic skeletal diseases, driven to osteogenic differentiation, could also be used in the in vivo transplantation model to study pathogenetic mechanisms and potential treatments. In this regard, there are two possible approaches: one would be in which iPSCs are derived from $CD34^+$ peripheral blood cells of patients with inherited forms of skeletal disease,[91] and then gene edited; or that a specific mutation be created by gene editing in normal iPSCs. The point of these two approaches is that the normal and the mutated lines would be isogeneic, and free of the potential confounding variable of genetic background. This feature may also allow for the determination of the contribution of a specific gene in polygenic diseases, characterized by the contribution of independent genes on generation of the phenotype. While a number of studies have been published showing osteogenic differentiation by in vivo transplantation, most are not robust, and more work is needed.[92] In addition, to date, none of the studies have shown that the osteogenic cells derived from iPSCs are able to support the formation of marrow, but this is not surprising based on the fact that bone comes before BM stroma during development. It may be that one would have to "educate" osteogenically differentiated IPSCs by coculture with endothelial cells to form an SSC/BMSC-like phenotype.

6 REGULATION OF SSC/BMSC FATE

As described previously, embryonic mesenchyme, which contains the most primitive SSC, forms cartilage, bone, bone marrow stroma, and marrow adipocytes in a sequential fashion in time and space (Fig. 15.2). Thus, during fetal development, chondrocytes, osteoblasts, SSCs/BMSCs, and marrow adipocytes can be seen as different stages of maturation rather than separate lineages.[15] In postnatal life, SSCs are quiescent due to their association with blood vessels until liberated by the need for bone turnover (Fig. 15.4). During fetal and postnatal life, chondrogenesis, osteogenesis, adipogenesis are controlled by three master transcription factors: Sox 9 (chondrogenic),[93] Runx2 (osteogenic and chondrogenic, but to a lesser extent),[94] and PPARγ2 (adipogenic) (reviewed in Ref. 95). While postnatal SSCs/BMSCs do form cartilage in vitro, it is extremely rare that they form cartilage in vivo (except in pathological conditions), or following injury. Fracture repair is mediated primarily by periosteal cells that are similar to, but not identical with SSCs/BMSCs[43] (reviewed in Ref. 96). For this reason, a chondrogenic cell fate of postnatal SSCs/BMSCs will not be discussed later.

Upon liberation from blood vessel walls, SSCs have the possibility to form osteogenic cells, adipogenic cells or to form more hematopoiesis-supportive stroma (Fig. 15.4). The balance between osteogenic and adipogenic differentiation has received a large amount of attention due to its relevance to bone fragility and osteoporosis.[97–101] On the other hand, there is little information on how the SSC is directed into becoming a hematopoiesis-supporting stromal cell that is not a stem/progenitor cell. There are clear examples of the expansion of the hematopoiesis, which relies on the presence of stroma, such as in hemolytic diseases where bone is lost to accommodate increased blood formation. However, it has rarely been determined if the increase in hematopoiesis-supportive BMSCs is matched by an increase in SSCs.

Regulation of cell fate is complex, and is controlled by many factors, including hormones, cytokines, growth factors, and their downstream signaling pathways, all of which impact on the epigenetic pattern of the genome and subsequent transcriptional activity. Further control arises from posttranscriptional modifications (splice variants and isoforms), translational variation (small noncoding RNAs), and posttranslational modifications (reviewed in Ref. 99). Mechanical forces, cell–matrix and cell–cell interactions also are influential. Lastly, there are a number of iatrogenic (illness caused by medical examination or treatment) and distantly related causes of fate switching. What follows in subsequent sections is a brief summary (while most certainly not complete, appropriate articles, and reviews are cited). However, it must be noted that in sorting through the information that is available concerning

regulation of fate choices, there is much confusion based on the fact that it is considered by many that adipocytes from white or brown fat are equivalent to those in marrow, but they are not. Furthermore, it is assumed that factors that induce osteogenic differentiation of SSCs/BMSCs will induce osteogenic differentiation of adipose-derived "MSCs," and they are not, primarily because adipose-derived "MSCs" are not inherently osteogenic.

6.1 Hormonal Regulation

SSCs/BMSCs express receptors for many of the hormones that control skeletal growth and homeostasis, such as the PTH receptor, vitamin D receptor, glucocorticoid receptors, sex hormone receptors, thyroid hormone receptor, and so on. If and how the corresponding ligands of these receptors control SSC/BMSC cell fate has not been closely examined in many cases. However, there are a few instances in which hormonal control or changes in their downstream signaling pathways (briefly described later) do impact on the fate of SSCs/BMSCs. For example, in hyperparathyroidism, it is thought that the ensuing endosteal fibrosis is due to the enhanced proliferation of stromal progenitor cells that accumulate and partially differentiate into osteoblastic cells.[102] Along this same line, excess PTH signaling due to a constitutively activating mutation in the PTH/PTHrP receptor (H223R), expressed specifically in mature osteoblasts in mice, led to the formation of excessive medullary bone due to the proliferation and maturation of SSCs/BMSCs, at the expense of marrow stromal cells and adipocytes.[103] Interestingly, this phenotype resolved with age with the removal of excess bone and establishment of a marrow cavity composed of stromal cells that were not SSCs based on in vivo transplantation assays. On the other side of the coin, it has long been known that estrogen deficiency leads to expansion of adipocytes at the expense of hematopoiesis-supportive stroma (and bone), especially in females,[104] which is thought to reflect a commitment of SSCs/BMSCs to adipogenesis.[105] However, it is also possible that direct conversion of stromal cells into adipocytes contributes to this phenomenon. Likewise, glucocorticoid treatment in vivo leads to conversion of red marrow to yellow marrow, again thought to be due to a switch in fate of SSCs/BMSCs.[106] Paradoxically, a glucocorticoid, dexamethasone, is commonly used in medium used to osteogenically differentiate SSCs/BMSCs,[26] implying that other factors are at play in vivo that drive glucocorticoid-mediated adipogenic differentiation of SSCs/BMSCs.

6.2 Signaling Pathways and Transcription Factors

The levels of expression of Runx2, the master regulator of osteogenesis, and PPARγ, the master regulator of adipogenesis, are controlled by a number of signaling pathways activated by Wnts, members of the TGFβ/BMP superfamily, Notch, Hedgehogs, FGFs (and others), with extensive crosstalk between these pathways (reviewed in Refs. 99,107–109). Generally speaking these pathways increase Runx2 expression with a concomitant decrease in PPARγ, or vice versa, during commitment and differentiation of SSCs/BMSCs. Runx2 is the first upregulated gene during osteogenic commitment, and in turn, upregulates Osterix, which along with Runx2, is essential for osteogenesis. Adipogenesis is initiated by upregulation of C/EBPβ and δ, which in turn induces PPARγ2. PPARγ2 upregulates C/EBPα, which maintains PPARγ2 expression via a positive feedback loop (reviewed in Ref. 107).

Perhaps one of the most important signaling pathways that promotes osteogenesis of SSCs/BMSCs at the expense of adipogenesis is the Wnt/β−catenin pathway, which stimulates Runx2 expression, and inhibits C/EBPα, thereby inhibiting adipogenesis[110,111] (reviewed in Ref. 107). Members of the TGFβ superfamily also exert major influences mediated by pSmads 2/3, and have multiple effects on differentiation of SSCs/BMSCs. While TGFβs stimulate proliferation, they inhibit osteogenic differentiation[112] and adipogenic differentiation.[113] Additionally, BMPs play a major role in osteogenic commitment via pSmads 1/5/8 and Msx/Dlx homeoproteins to increase expression of Runx2 (reviewed in Ref. 107). However, BMPs also appear to play a role in early adipogenic commitment, although the mechanisms are not clear. Adipogenic differentiation may be related to the type of BMP and its concentration, and/or the type of receptors that are present on the cells at different stages of commitment (reviewed in Refs. 99,108). It is generally reported that Notch signaling mediated by Delta/Jagged suppresses osteogenic differentiation,[114] and maintains SSCs/BMSCs in a primordial state,[115,116] however, positive effects on osteoblastogenesis have been reported (reviewed in Refs. 108,109). Notch signaling also appears to have negative and positive effects during adipogenic commitment and differentiation (reviewed in Ref. 95). Indian hedgehog signaling, mediated by the Kinesin family protein, Kif7, and the main intracellular Hedgehog pathway regulator, SUFU, is typically thought of in the context of growth plate dynamics, where it promotes osteogenic differentiation.[117] However, in the context of the BM microenvironment, Hedgehog signaling decreases with adipogenic differentiation of SSCs/BMSCs, suggesting that it is a negative regulator of adipogenesis.[118] Hedgehogs may also interact with BMP signaling to promote osteogenic commitment (reviewed in Ref. 99). FGFs can also be osteogenic (FGF2, FGF4, FGF3, FGF9, and FGF19) and adipogenic (FGF1, FGF2, and FGF10), and signal via a number of pathways that include ERK1/2, p38 MAPK, JNK, PKC, and PI3K[119,120] (reviewed in Ref. 99).

As briefly listed previously, these are the major players that have implicated in controlling the fate of SSCs/BMSCs, but there are other pathways initiated by factors, such as IGF-1 and PDGF (liberated from bone matrix during bone resorption), EGF, and the transcription factor, YAZ (reviewed in Refs. 99,107–109). There are likely to be more regulators identified in the future.

6.3 Epigenetic Controls

It is currently thought that epigenetic changes induced by signaling pathways operating in SSCs/BMSCs play a major role in fate decisions and stabilization of the osteogenic or adipogenic phenotype.[97] Modification of the conformation of chromatin (defined as DNA with bound protein) impacts on transcription, with open chromatin (euchromatin) being accessible, and condensed chromatin (heterochromatin) being inaccessible to transcriptional machinery, transcription factors, and other cofactors. Changes in chromatin architecture are brought about by DNA and histone modification, reviewed very succinctly in the following website: http://www.sabiosciences.com/pathwaymagazine/pathways8/epigenetic-modifications-regulate-gene-expression.php.

DNA methylation at CpG dinucleotides and CpG islands by methyltransferases (DNMTs), are usually suppressive in nature. It has been reported that "MSCs" from different tissues have similar DNA methylation patterns,[98] but this is most likely due, in part, to the fact they share fibroblastic and/or pericytic features, and expression of common extracellular matrix proteins. More recently, it has been determined that "MSCs" from different tissues, including SSCs/BMSCs, can be distinguished from each based on an "Epi-Tissue Score" at two CpG sites.[121]

In addition to DNA methylation, chromatin structure is also influenced by modifications made to histones bound to DNA, such as methylation and acetylation (and there are others) that occur posttranslationally. Histones 3 and 4 are commonly methylated at specific sites by histone methyltransferases (HMTs). H3K4me3 is usually associated with euchromatin and gene activation, whereas H3K9me3 and H3K27me3 are usually associated with heterochromatin and gene repression. These methyl groups can be removed by histone demethylases (HDMs), and the changes in methylation are very dynamic, promoting flexibility. As an example, it has been demonstrated that formation of H3K27me3 by the HMT, EZH2, promotes adipogenesis of SSCs/BMSCs, whereas its removal by the HDM, KDM6A, promotes osteogenesis.[122] Histone acetylation by histone acetyltransferases (HATs) and deacetylation by deacetylases (HDACs) is also dynamic. H3K9ac and HRK16ac are generally found in euchromatin and are indicative of transcriptional activation, whereas deacetylation leads to gene inactivation. While use of the HDAC inhibitor, trichostatin A, was thought to improve osteogenic differentiation of SSCs/BMSCs, it was determined that it profoundly decreased cell proliferation, and did not improve their osteogenic capacity upon in vivo transplantation.[123] Further work is needed to demonstrate what role histone acetylation and deacetylation plays in SSC/BMSC cell fate.

6.4 MicroRNAs

There are three forms of small noncoding RNAs: shorthairpin RNAs (shRNAs), microRNAs (miRs), and piwi RNAs. shRNAs are generally synthetic, and introduced exogenously (or created by processing of foreign dsRNAs), and piwi RNAs are primarily expressed in germline cells (reviewed in Refs. 124,125), consequently, the role of miRs in controlling cell fate will be briefly summarized. miRs represent a way in which protein expression can be rapidly modified by either blocking translation or inducing the degradation of target mRNAs (reviewed recently in Ref. 126). Interestingly, regulators of skeletal homeostasis, such as BMPs and TGFβs are known regulators of miR expression and processing (reviewed in Ref. 127). Numerous studies have been published indicating that miRs can control cell fate by downregulating the protein level of Runx2 or PPARγ, thereby enhancing adipogenesis or osteogenesis, respectively. Members of the miR-320 family were found to increase during adipogenic differentiation of SSCs/BMSCs by decreasing levels of Runx2 (among other genes).[128] Conversely, mir-20a promotes osteogenesis by targeting PPARγ, as well as inhibitors of the BMP signaling pathway.[129] In addition, mir26a was found to decrease in SSCs/BMSCs during rapid bone loss in mice induced by ovariectomy. Overexpression of mir26a was found to increase osteogenesis by SSCs/BMSCs by reducing levels of Tob1, which is a negative regulator of the BMP/Smad signaling pathway.[130] These are just few examples, and others can be found in a recent review.[131]

6.5 Cell–Cell and Cell–Substrate Interactions, Cell Shape, and Mechanical Forces

SSCs/BMSCs are not alone in the bone/marrow organ, and interaction with other cell types undoubtedly influences fate. As already described previously, interaction with endothelial cells keeps them in a primordial state, and a study has shown that this is due to repression of Osterix.[132] They also interact with hematopoietic stem cells; however, how HSCs and other hematopoietic cells influence SSC fate is not well known at this time, owing to the rather recent recognition by hematologists that SSCs are members of the HSC niche (reviewed in Ref. 133).

Cell–matrix interactions occur primarily through: (1) integrins (which bind to many of the extracellular matrix components of bone), (2) discoidin domain receptors (DDRs) and urokinase plasminogen activator receptors (uPARAP), which bind to collagens, and (3) other cell surface molecules, such as CD44, which binds to Osteopontin (a major component of bone matrix), and hyaluronan. Of note, BM is characterized by a preponderance of cell–cell interactions rather than cell–matrix interactions. Marrow contains thin reticular fibers composed primarily of type III collagen, and high levels of hyaluronan, which is gel-like in nature (reviewed in Ref. 134). However, there is no doubt that, after isolation from marrow, substrate has a profound influence on cell fate. For example, binding of αvβ1 to Osteopontin inhibited adipogenic differentiation and stimulated osteogenic differentiation of murine SSCs/BMSCs, via downregulation of C/EBP expression.[135] Modulation of cell shape also impacts on fate. In a study where cell shape was controlled by plating at different densities, it was found that if cells were plated at low density and allowed to spread, the cells were directed toward osteogenesis, whereas plating at high densities, such that the cells remained rounded, induced adipogenesis. These phenomena were found to be mediated by the small GTPase, RhoA, which is a regulator of the cytoskeleton.[136] Currently there are a number of different methods by which substrates can be patterned, not only in shape, but in their surface character.[137] Patterns that promoted cytoskeletal contraction via JNK and ERK1/2 activity induced osteogenic differentiation, whereas when cytoskeletal contraction was not supported by a particular pattern, adipogenesis predominated.[138] Substrate elasticity and stiffness, and the resulting mechanical forces sensed by SSCs/BMSCs control fate as well, with stiffer substrates generally favoring osteogenesis, and softer substrates favoring adipogenesis. How substrate interactions control cell fate is extensively reviewed in Ref. 139. However, again the reader is cautioned that many of these studies purport differentiation of SSCs/BMSCs into nonskeletal phenotypes based on morphology and expression of a few markers, without demonstration of functionality. Lastly, matrix remodeling has a profound effect on the fate of SSCs/BMSCs. A dramatic example is found in the global deletion of the membrane type matrix metalloproteinase, MT1-MMP (which is a true collagenase), in mice. Although mice appear normal at birth, there is a rapid decrease in growth and by 40 days they exhibit severe dwarfism and multiple skeletal defects. Transplantation of SSCs/BMSCs from the mutant mice revealed an almost complete inability to reform a bone/marrow organ, indicating that MT1-MMP activity is essential for SSC self-renewal.[140] This mouse model is a direct phenocopy of the human vanishing bone disease, Winchester syndrome, now known to be caused by an inactivating mutation in MT1-MMP.[141]

6.6 Iatrogenic Effects

In addition to all the aforementioned, iatrogenic effects (i.e., caused by medication or other forms of therapy) and seemingly unrelated diseases can impact on SSC/BMSC fate. Chemotherapy and radiation therapy are well known to induce marrow adiposity (reviewed in Ref. 105). In addition, there is a conversion of red marrow to yellow marrow in extreme cases of starvation, as in anorexia nervosa.[142] Of interest, there is a striking conversion of yellow marrow to red marrow in many patients with congestive heart failure.[143]

7 SSCs/BMSCs IN TISSUE ENGINEERING AND REGENERATIVE MEDICINE

7.1 Regeneration of the Bone/Marrow Organ

Generation of sufficient numbers of SSCs/BMSCs to reconstruct a critical size bone defect that will never heal on its own is an area of intense research (Fig. 15.6A), along with development of optimal scaffolds and surgical approaches. As aforementioned, it has been shown that the SSCs reside in marrow on the abluminal side of marrow sinusoids.[12] Consequently, if a population of BMSCs is found to form only bone upon in vivo transplantation, it is indicative of the fact that the SSCs within the population have been lost. Thus, the presence of marrow in in vivo transplants of BMSCs is a surrogate marker of presence of the SSCs, and of the ability of that bone/marrow organ to undergo bone turnover and remodeling.

However, one may ask why is it necessary to maintain SSCs in the BMSC population; would more committed osteogenic cells not suffice? The answer most likely is that sometimes they would and sometimes they would not, depending on the rate of bone turnover. In areas of the skeleton that turnover very slowly and are not weight bearing (e.g., the skull vault), the use of more mature populations of cells may be sufficient. In addition, regeneration of bone with mature osteogenic cells that have lost the ability to self-renew may be efficacious in older recipients in nonweight-bearing bones. However, in cases where higher levels of bone formation and remodeling are required (e.g., in weight-bearing bones), SSCs are a necessary ingredient in any type of construct for long-term efficacy. In younger recipients, the ability to turn over throughout life is essential to maintain bone strength, and the engineered tissue construct must contain a stem/progenitor cell.

In protocols for bone regeneration, there are additional considerations: (1) autologous (self) versus allogeneic (nonself); and (2) embryonic origin of the bone. It is thought that SSCs/BMSCs are immune privileged; that is, that they can escape rejection when used in an allogeneic setting. This may be the case when allogeneic

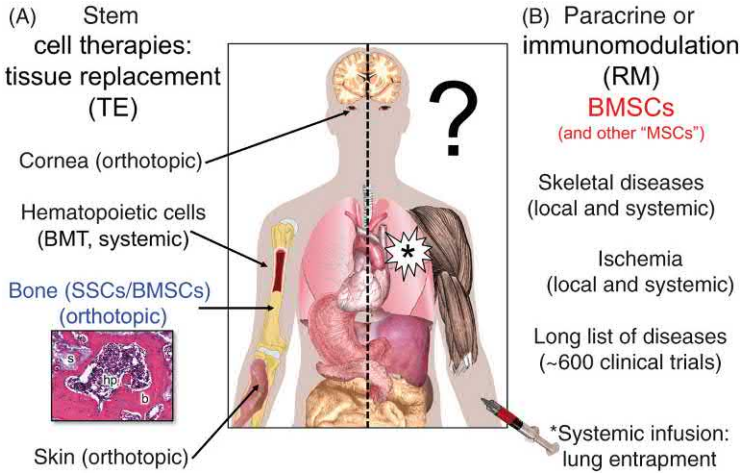

FIGURE 15.6 The role of SSCs/BMSCs in tissue engineering (TE) and regenerative medicine (RM). A. There are a very limited number of cases in which tissue-specific stem/progenitor cells themselves have been successfully used to regenerate a tissue (limbal stem cells to regenerate cornea, hematopoietic stem cells in BM transplantation, and epidermal stem cells for skin regeneration. SSCs/BMSCs have been demonstrated to regenerate functional bone in small number of case reports,[144] and efforts are continuing to the development of direct orthotopic procedures with appropriate scaffolds. B. On the other hand, there is a great deal of effort to use SSCs/BMSCs (and other types of "mesenchymal stem/stromal cells") for generalized skeletal diseases and a long list of other diseases and disorders by systemic infusion or direct injection without a carrier (over 600 clinical trials, see clinicaltrials.gov). This is based on their putative paracrine and immunomodulatory effects, whereby they encourage local stem/progenitor cells to begin the repair process. However, these cells are rapidly trapped in the lungs and disappear, and it is not clear what the mechanism of actions is for these types of treatments.

or xenogeneic BMSCs are introduced into developing embryos prior to establishment of the immune system.[145] However, there are studies that show that allogeneic and xenogeneic BMSCs are indeed rejected when infused systemically into immune-competent recipients.[146] They are even more likely to be rejected when they are induced to differentiate, based on their expression of histocompatibility antigens. Consequently, for bone TE, cells will most likely have to be autologous in order to avoid the need for immunosuppression of the recipient. In addition, the source of SSCs/BMSCs may need to be considered. As aforementioned, bones in the facial region of the skull derive from neuroectoderm, while those in the axial and appendicular skeleton derive from mesoderm (reviewed in Ref. 16). There are studies indicating that cells from these two different embryonic sources are not identical.[147] Oral surgeons have long noted that transplantation of iliac crest (mesodermally derived) into the jawbones (neuroectodermally derived) is not enduring,[148] suggesting some sort of incompatibility. Far less is known about differences between bone formed by paraxial mesoderm (axial skeleton) and by somatic lateral plate mesoderm (appendicular skeleton), and it is not clear if this impacts on their use in sites outside of their developmental origin.

7.2 SSCs/BMSCs in Another Form of Regenerative Medicine

While not directly related to the topic of this book, it cannot be ignored that SSCs/BMSCs are being used in a different form of regenerative medicine (RM) that does not utilize SSCs/BMSCs in conjunction with appropriate scaffolds to reform bone (TE). BMSCs (as well as other types of "MSCs") are being used in a large number of clinical trials around the world (see clinicaltrials.gov) for treatment of a variety of skeletal and nonskeletal diseases and disorders (Fig. 15.6B) caused by systemic infusion or direct injection. What has become apparent in preclinical animal studies is that upon systemic infusion, these cells are rapidly cleared from the circulation by entrapment in the lungs. Even upon direct orthotopic injection (without a scaffold), the cells do not readily engraft and rapidly disappear. Nonetheless, animal studies suggest that there may be a beneficial effect for treatment of skeletal diseases and disorders, perhaps due to the production of cytokines and growth factors secreted by the donor cells that encourage local stem/progenitor cells to begin a repair process (albeit at a distance when the cells are trapped in the lungs); the so-called paracrine, immunomodulatory, and immunosuppression effects.[19,149,150] It is also thought that locally administered cells may stabilize blood vessels (again, going back to their pericytic nature). Whatever the potential mechanisms of action are, it is clear that this is a form of RM that is not stem cell based. Rather, it relies on the activity of the BMSC population as a whole, not on the subset of SSCs within it, which is relatively rare. Furthermore, it may be that all cells of fibroblastic habit (MSCs) have similar effects,[151] although their effects may be a variation on a theme based on their tissue source. To date, it is not

clear whether this type of cell-based therapy will prove to be efficacious.

8 CONCLUSIONS

The bone/marrow organ is a complex system that includes not only hard tissue proper, but also a population of bona fide SSCs and more committed BMSC progenitor cells. These cells are quite unique in their ability to not only form the specialized mineralized matrix of bone, but also in their support of hematopoiesis, a property that is not found anywhere else in the normal postnatal organism. These cells are not like other types of "MSCs" based on these exclusive properties. Likewise, nonskeletal "MSCs" are not like SSCs/BMSCs, and for these reasons, the terminology should be abandoned in favor of names of cells that reflect their tissue of origin, and their status as a stem or progenitor cells, based on rigorous assays.

SSCs/BMSCs arise from different specifications of neural crest and mesoderm, and during development, they are formed by the incorporation of osteogenically committed cells into blood vessel walls as pericytes. SSCs/BMSCs remain quiescent until stimulated by factors signaling for a bone turnover event, in which SSCs/BMSCs control, at least in part, the formation and activity of osteoclasts, and replace resorbed bone. SSCs/BMSCs also respond to changes in the microenvironment, and can adopt an osteogenic, adipogenic, or stromal cell fate. SSC/BMSC cell fate is controlled by many extrinsic factors (hormones, growth factors, cytokines), mediated by a multitude of signaling pathways, but also intrinsic factors (mutations, epigenetics) that alter the biological activity of SSCs/BMSCs. Taken together, one could consider SSCs/BMSCs to be the "units" of skeletal diseases, akin to Koch's postulates to identify the causative agent of a particular disease: (1) the microorganism or pathogen (the mutated cell, in this case) must be present in all cases of the disease, (2) the pathogen (mutated cell) can be isolated from the diseased host and grown in pure culture, (3) the pathogen from the pure culture must cause the disease when inoculated into a healthy, susceptible laboratory animal (in vivo transplantation into mice to recapitulate the formation of an abnormal bone/marrow organ), (4) the pathogen must be reisolated from the new host and shown to be the same as the originally inoculated pathogen (self-renewal of the phenotype, or of the stem cell). Thus, generating ectopic ossicles with populations of normal and mutated SSC/BMSC populations offers a unique model system for the study and treatment of genetic diseases of the skeleton. While mouse models of disease are of interest, there are cases in which a known mutation in man does not create a phenocopy when introduced into mice.

In addition to serving as a model system for study skeletal homeostasis and disease, the remarkable ability of SSCs/BMSCs to recreate a bone/marrow organ themselves (when transplanted with an appropriate scaffold) makes them a leading candidate for bona fide TE. However, there are many hurdles yet ahead in terms of appropriate delivery and stabilization strategies, based on the time required to generate functional weight-bearing bone. While many have hoped that these cells would be useful for treatment of systemic skeletal diseases and disorders by systemic infusion or direct injection due to paracrine, immunomodulatory, and immunosuppressive effects of the cells, this is not a stem cell therapy. Nor are there clear mechanisms of action based on the fact that the cells rapidly disappear. More preclinical studies that identify rigorous outcome parameters to be measured and establishment of efficacy are needed. These are not only exciting times in the area of stem cell research, but also a time in which clinical applications should proceed with caution. There is much to be learned about tissue-specific stem/progenitor cells; what they are, and what they are not, and what they can and cannot do. Nonetheless, SSCs/BMSCs offer a unique model system for study of genetic and acquired diseases of the skeleton, and development of novel therapeutic strategies.

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CHAPTER

16

Osteoimmunology

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1 INTRODUCTION

Bone is a key component of the skeletal-locomotor system and serves as a calcium reservoir regulated by the endocrine system in vertebrates.[1] Bone also functions as the "primary lymphoid organ" in that it harbors hematopoietic stem cells (HSCs), immune progenitor cells, and mature immune cells, including B cells. The immune cells and bone cells share not only the same microenvironments in the bone marrow, but also a number of regulatory molecules, including cytokines, receptors, signaling molecules, and transcription factors. The systems interact with each other to cooperatively carry out the functions of bone. "Osteoimmunology" was created as an interdisciplinary field to investigate the shared mechanisms and inseparable link between the bone and immune systems.

The immune system first emerged in primitive animals and plants, subsequently developing into the complex system of innate and adaptive immunities in vertebrates. The highly sophisticated immune system in vertebrates requires functionally specialized immune cells, as well as the tissues in which these cells develop and activate, such as the thymus, lymph nodes, and bone marrow. The fact that bone and the acquired immune system appeared at the same stage of vertebrate evolution makes us speculate that the immune system included the bone as a part of its essential units during the course of evolution.[2] The cells in the bone marrow are largely divided into two types, cells engaged in bone metabolism and hematopoietic cells. By interacting with each other, these cell types cooperatively carry out the required functions of the bone, such as body support, control of mineral metabolism, and hematopoiesis.[3] Now the situation is such that it has become necessary to keep this "osteoimmune system" in mind whenever we think about anything related to either system.

The close relationship between the immune and bone systems has been appreciated since the pioneering studies on immune cell-derived osteoclast-activating factors in the 1970s.[4,5] The term osteoimmunology was coined in 2000 to highlight the reciprocal interplay between these two systems.[6,7] Subsequently, studies on the bone destruction associated with rheumatoid arthritis (RA), as well as the identification of the various bone phenotypes found in genetically modified, immunocompromised animals have further unveiled the physiological and pathological significance of the shared mechanisms and cross talk between the two systems. It is now well known that numerous immune cytokines influence bone cell activity and bone mass. Among them, receptor activator of NF-κB ligand (RANKL) is one of the most important molecules that explicitly link the two systems. RANKL was first cloned as a member of the TNF superfamily expressed by T cells in order to stimulate dendritic cells (DCs),[8,9] and was rediscovered as an essential factor for osteoclast differentiation.[10,11] RANKL has been shown to serve multiple immune functions. Moreover, the immune regulation of osteoclasts is closely associated with the pathogenesis of RA, which is characterized by a progressive destruction of multiple joints. The bone destruction in RA is the result of the enhanced osteoclast activity due to the inflammatory responses triggered by the activation of a unique helper T cell subset, Th17 cells.[12,13] As evidenced by the clinical benefits conferred by anti-TNF and anti-IL-6 therapy on inflammation and osteoclast differentiation in RA, the osteoimmunological approach is now of growing importance in clinical applications. Furthermore, with the intense global competition in the research field of the hematopoietic stem cell niche, the physiological significance of bone as a "primary lymphoid organ" has been underscored. This chapter provides an overview of recent progress in the field, as well as an overview that frames a context for the basic and clinical investigation of the skeletal and immune systems.

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2 THE RANKL/RANK SYSTEM IN BONE METABOLISM

2.1 RANKL is Essential for Osteoclastogenesis

A relationship between the immune and bone systems has been suggested since the original pioneering studies on the osteoclast biology in the 1970s. Soluble factors produced by antigen-stimulated immune cells, such as interleukin (IL)-1, were shown to stimulate osteoclastic bone resorption.[4,14,15] In the 1980s, studies using in vitro culture systems for osteoclast formation indicated that the osteoblast lineage cells or the bone marrow stromal cells of the mesenchymal lineage are involved in the induction of osteoclastogenesis. Burger et al. showed in vitro osteoclast formation using a coculture of murine hematopoietic cells and embryonic bone rudiments containing osteoblasts, chondrocytes and osteocytes.[16] Takahashi et al. established another in vitro coculture system for osteoclastogenesis that has come into general use. This coculture system required cell-to-cell contact between the bone marrow-derived cells of monocyte/macrophage lineage and the osteoblast lineage cells derived from calvaria.[17] It was thus suggested that osteoclastogenesis-supporting cells must provide an osteoclast differentiation factor (ODF).[18] Analysis of *op/op* mice with osteopetrosis revealed macrophage colony-stimulating factor (M-CSF) to be one of the factors essential for osteoclastogenesis. However, M-CSF alone does not induce the differentiation of osteoclasts. One year after the cloning of the inhibitor of osteoclastogenesis, osteoprotegerin (OPG; encoded by the *Tnfrsf11b* gene),[19,20] Yasuda et al. and Lacey et al. independently identified ODF and OPG ligand, respectively, as the long-sought ligand for osteoclast differentiation.[10,11] Interestingly, this cytokine, which belongs to the tumor necrosis factor (TNF) family, was shown to be identical to RANKL (encoded by the *Tnfsf11* gene) and TNF-related activation-induced cytokine (TRANCE), both of which had been cloned as T cell cytokines in the field of immunology.[8,21] The receptor for RANKL is RANK (encoded by the *Tnfrsf11a* gene), a type I transmembrane protein.[8,22] OPG acts as a soluble decoy receptor that blocks RANK signaling by binding to RANKL with a higher affinity.[10,11] Mice with a disruption of either the *Tnfsf11* or *Tnfrsf11a* gene exhibit severe osteopetrosis accompanied by a tooth eruption defect owing to a complete lack of osteoclasts.[23–25] Mice lacking the *Tnfrsf11b* gene exhibit a severe form of osteoporosis due to an increase in both the osteoclast number and bone resorption.[26–28] In humans, mutations in *TNFRSF11A*, *TNFSF11*, and *TNFRSF11B* cause bone disorders, such as familial expansile osteolysis, autosomal recessive osteopetrosis, and juvenile Paget's disease, respectively.[29–33] These genetic findings provide clear evidence that the RANKL/RANK signaling is essential for osteoclast differentiation.

RANKL is expressed as two forms: a membrane-anchored and a secreted protein form. The soluble form arises from proteolytic processing of the membrane form, by metalloproteinases, such as matrix metalloproteinase (MMP)-14.[34,35] Although both forms function as agonistic ligands for RANK, the membrane-bound RANKL is more efficient.[34–36] In bone, RANKL is expressed by several different mesenchymal cells, including osteoblasts, chondrocytes, and osteocytes in response to certain osteoclastogenic factors, such as 1,25-dihydroxyvitamin D_3, prostaglandin E_2, parathyroid hormone (PTH), IL-1, IL-6, and IL-17.[34,37]

Certain reports have suggested that osteoclasts can differentiate independently of RANKL.[38–41] TNF-α), APRIL, BAFF, NGF, IGF I, IGF II, LIGHT, TGF-β, IL-6, IL-11, and SOFAT have been proposed as RANKL-independent osteoclastogenic factors. However, most of these reports lack clear experimental evidence showing that RANKL can be replaced by other molecules, even under RANKL- or RANK-deficient conditions. Kim et al. showed that TNF-α plus TGF-β induces osteoclastogenesis in RANK or RANKL-deficient cells in vitro, but the in vivo effects were not evaluated.[39] Administration of high dose TNF-α into RANKL-deficient mice leads to a minimal local formation of tartrate-resistant acid phosphatase (TRAP)-positive cells, but not functional osteoclasts.[25] It has been recently reported that lysyl oxidase (LOX)-induced osteoclastogenesis in vitro without any addition of recombinant RANKL.[40] However, LOX failed to rescue the in vitro osteoclastogenesis or osteopetrotic phenotype of RANKL-deficient mice.[42,43] Therefore, RANKL-independent osteoclastogenesis has never been demonstrated thus far in vivo and there is no molecule that substitutes for RANKL in osteoclastogenesis. RANKL-independent osteoclastogenesis should properly be judged using RANKL/RANK-deficient conditions in order to unambiguously exclude the involvement of permissive levels of RANKL.

2.2 The Cellular Source of RANKL in Bone Remodeling

RANKL is expressed by various types of mesenchymal cells, including osteoblasts, osteocytes, and hypertrophic chondrocytes.[44–47] Although osteoblasts and bone marrow stromal cells (BMSCs) have been considered the major source of RANKL in vivo,[7,18] recent studies using RANKL conditional knockout mice has further revealed the physiological relevance of each cell type-derived RANKL on bone metabolism. In addition, the development of a method for isolating highly pure osteocytes from bone tissue using osteocyte-specific reporter mice has further advanced our knowledge of the characteristics of osteocytes.[47] Isolated osteocytes were found to express the *Tnfsf11* mRNA at a higher level and have a greater capacity to induce osteoclastogenesis

in vitro than isolated osteoblasts.[47] Osteocyte-specific RANKL-deficient mice, which were generated by crossing *Tnfsf11*-floxed mice with *Dmp1-Cre* mice, were born without any obvious skeletal defect, but became osteopetrotic postnatally due to a severe reduction in osteoclast number.[47] The age-dependent osteopetrotic phenotype observed in osteocyte-specific RANKL-deficient mice indicates that osteocyte-derived RANKL plays a key role in bone remodeling after birth, but not skeletal development in the embryo. Osteocytes are embedded in the bone matrix and are thought to act as mechanosensory cells. In a mechanical stress experiment using osteocyte cell line MLO-Y4 cells, RANKL expression was significantly induced by mechanical loading.[47] In addition, unloading-induced bone loss was prevented in osteocyte-specific RANKL-deficient mice (generated with *Dmp1-Cre*).[48] Thus, osteocytes are suggested to sense mechanical stress and regulate the expression level of RANKL.

The importance of osteoblasts as a source of RANKL in bone remodeling after birth has been evaluated using inducible deletion systems of osteoblasts in adult mice.[48] Inducible ablation of cells expressing a thymidine-kinase transgene under the control of osteocalcin promoter by gancyclovir administration led to a defect in bone formation but exerted no effect on osteoclastic bone resorption.[49] A similar approach using type I collagen promoter-thymidine-kinase transgenic mice also showed that the deletion of osteoblasts did not affect RANKL expression in bone tissue.[50] Furthermore, inducible deletion of *Sp7* (encoding Osterix)-expressing cells, which are considered to be committed osteoblast progenitors, did not reduce the number of osteoclasts. Therefore, it is unlikely that the RANKL on osteoblasts in adults contributes much to bone remodeling. On the other hand, in *Tnfsf11*-floxed mice crossed with *Prx1*-Cre mice, which express *Cre* recombinase in mesenchymal progenitors in the developing limbs, including all chondrocytes, osteoblasts, and osteocytes, exhibit severe limb bone osteopetrosis at birth.[48] Conditional deletion of RANKL in chondrocytes using type X collagen Cre mice resulted in a severe osteopetrotic phenotype at birth.[48] A similar bone phenotype was observed in *Tnfsf11*-floxed mice crossed with *Sp7*- or *Bglap*-Cre mice. Therefore, the RANKL expressed by osteoblasts and chondrocytes contributes to the osteoclastogenesis that occurs in fetal skeletal development.[48]

Several Cre transgenic mouse lines were generated in order to specifically target and analyze osteocytes. On the basis of the specific expression of Dmp1 in osteocytes, 10 kb-*Dmp1-Cre*, 8 kb-*Dmp1-Cre* mice, and mice expressing tamoxifen-inducible Cre under the control of the 10 kb *Dmp1* promoter were generated as osteocyte-specific Cre mice.[51–53] Although 10 kb-*Dmp1-Cre* mice have been widely used in osteocyte-specific deletion, Cre recombination was reported to be observed in unexpected cell types when the 10 kb-*Dmp1* Cre mice were crossed with the reporter mice, such as mature osteoblasts, CXC chemokine ligand (CXCL) 12-abundant reticular (CAR) cells, and muscle cells.[54,55] It should be noted that Dmp1 expression was also reported in brain, pancreas, and kidney.[56] *Sost-Cre* transgenic mice are also accepted as being specific for osteocytes, but not bone-forming osteoblasts, among bone cells.[57] Mice lacking RANKL in osteocytes using *Sost-Cre* mice also exhibit a severe osteopetrotic phenotype similar to that of the RANKL conditional knockout mice generated using *Dmp1-Cre* mice.[57]

Although RANKL was originally identified in activated T cells, the role of RANKL on T cells has been highlighted mainly in the context of autoimmune arthritis.[7] T-cell specific expression of RANKL partially rescued the osteopetrotic phenotype of RANKL-deficient mice,[58] implying that the RANKL on T cells has the capacity to regulate bone remodeling under physiological conditions. However, neither T cell-specific nor B-cell specific *Tnfsf11*-deficient mice exhibit any discernible osteopetrotic phenotype.[47,59] Furthermore, transplantation of HSCs from a healthy donor did not improve the osteopetrotic phenotype of humans with a mutation in the *TNFSF11A* gene.[29] Thus, overall, the RANKL expressed on T cells does not contribute to the physiological regulation of osteoclastogenesis.

2.3 The Role of RANKL in the Mammary Gland and Cancer

Mice lacking the *Tnfrsf11a* or *Tnfsf11* gene fail to form lactating mammary glands, resulting in the death of newborn pups. RANKL and RANK on mammary epithelial cells are essential for development of the lobulo-alveolar mammary structures during pregnancy.[60] The RANKL signaling in mammary epithelium is also involved in progesterone derivative-driven mammary cancers.[61,62] Mammary stem cell activation by RANKL signaling is crucial for the oncogenic activity of both estrogen and progesterone.[63,64] Moreover, recent studies have shown a key role of the RANKL/RANK axis in *BRCA1*-associated breast cancer and also the therapeutic potential value of RANKL inhibition.[65,66] The RANKL provided by tumor-infiltrating regulatory T (Treg) cells stimulates the metastatic progression of RANK-expressing mammary carcinoma cells.[67]

Bone is a common metastasis site for certain cancers, including breast, prostate, and lung cancers.[68] Bone metastases result in pain, fracture, spinal cord compression, and hypercalcemia, but the mechanism underlying the preferential metastasis to bone is not well elucidated. RANKL is important for cancer progression in bone through its effect in activating osteoclastic bone

resorption. The anti-RANKL neutralizing antibody Denosumab has attracted attention because it helps prevent skeletal-related events in breast cancer patients more efficiently than bisphosphonates.[69] RANKL has been reported to act as a chemotactic factor, recruiting certain epithelial tumor cells and melanoma cells that express RANK to bone.[70] The metastasis of prostate cancer to bone is also related to the RANKL/RANK system. Tumor-infiltrating RANKL-expressing inflammatory cells promote prostate cancer metastasis by inhibiting the expression of Maspin, a metastasis suppressor.[71] These findings show that the RANKL/RANK system plays a critical role in the metastatic behavior of cancer cells.

3 IMMUNOLOGICAL ROLE OF THE RANKL/RANK SYSTEM

The TNF superfamily of cytokines is known to be involved in a diverse range of immunological responses and immune organ development.[72] The RANKL/RANK system is not limited to the regulation of bone remodeling. As mentioned earlier, RANKL was originally identified as a T cell–derived cytokine. RANK is now known to be expressed at high levels in lymphoid tissues, breast epithelium, hematopoietic cells, and epithelial cells.[11,23,73] The RANKL/RANK system is essential for the development of mammalian immune organs, such as the thymic medulla and lymph node.[23,74,75] Considering that the bone marrow space is created by osteoclastic bone resorption, it can be said that RANKL is a cytokine critical for immune organ development in vertebrates (Fig. 16.1). Animals with hard bones received benefit of extended mobility while also incurring the increased risk associated with an exposure to a much more diverse array of pathogens, which one would expect to be an important driver in the emergence of a highly organized immune system. The immune organs can be divided into primary and secondary lymphoid tissues. The primary lymphoid tissues, where lymphocytes develop from progenitor cells and mature, are the bone marrow and thymus. The secondary lymphoid tissues are sites of lymphocyte activation and proliferation, and are comprised of the lymph nodes, spleen, and other gut-associated lymphoid tissue (GALT) in mammals. From analyses of mice lacking the *Tnfrsf11a* or *Tnfsf11* gene, the RANKL/RANK system has been shown to be essential for the development of mammalian immune organs, such as the thymus and lymph nodes.[23,74,75]

The thymus provides a specialized microenvironment that is maintained by cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs), for regulation of the selection of a functional and self-tolerant T-cell repertoire.[76,77] In the thymic cortex, $CD4^+CD8^+$ (double positive) thymocytes expressing T-cell antigen receptors (TCRs) are selected for survival or death depending on the avidity of the interaction between the TCR and the self-peptide/MHC (pMHC) complexes presented by cTECs. A weak avidity pMHC–TCR interaction induces the survival and differentiation into $CD4^+CD8^-$ or $CD4^-CD8^+$ single-positive (SP) thymocytes. After their migration into the thymic medulla, positively selected SP thymocytes are further screened for self-reactivity via interactions with mTECs.[78] mTECs express a variety of peripheral tissue-specific antigens, a process which is controlled by the unique nuclear proteins autoimmune regulator (Aire) and forebrain-expressed zinc finger 2 (Fezf2).[79–82] SP thymocytes that recognize the pMHC expressed by mTECs are destined to die. Thus, mTECs are essential for the deletion of the self-reactive T cells that initiate autoimmune diseases. RANK is highly expressed in mTECs and is required for Aire-expressing mTEC development.[74,83] In mice deficient

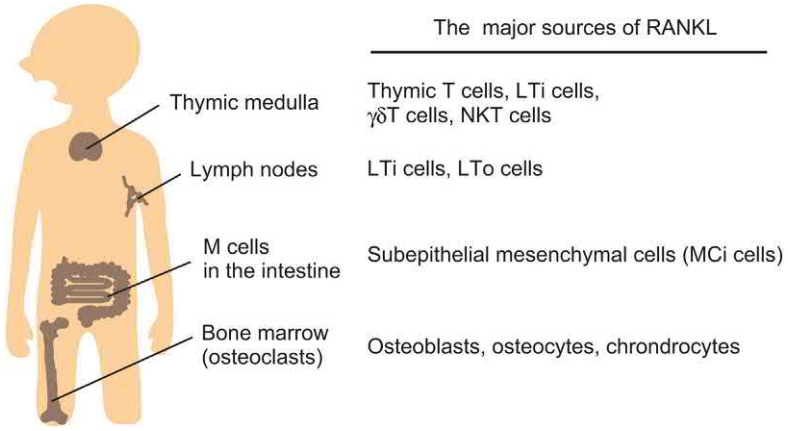

FIGURE 16.1 The RANKL-RANK system in the development of the immune organs. RANKL is a cytokine essential for the differentiation of osteoclasts, the activity of which causes the formation of the bone marrow cavity. In this sense, RANKL plays an essential role in the development of the immune organs in vertebrates, including the bone marrow, thymus, lymph node, and GALT.

in either RANK or RANKL, Aire-expressing mTECs are markedly reduced.[74,75,83] Moreover, the transplantation of RANK-deficient thymic stroma into nude mice induces signs of autoimmunity.[74,75] Various cell types in the thymus produce RANKL so as to promote the development of the mTECs that express RANK. Positively selected SP thymocytes are the most prominent source of RANKL in the postnatal thymus.[83–85] In addition, LTi cells,[74,83,85] γδT cells,[83,86] and natural killer T (NKT) cells[87] express RANKL and cooperate to maintain the thymic medullary microenvironment during ontogeny. The OPG expressed by mTECs acts as a negative feedback regulator of RANKL-RANK signaling in the thymus.[83,88] mTEC development is also controlled by the interaction between CD40L by CD4 SP thymocytes and CD40 on mTECs.[83] Mice doubly deficient for CD40 and RANKL exhibit an almost complete loss in Aire-expressing mTECs, whereas CD40-deficeint mice display only a mild defect.[75] Collectively, the RANKL-RANK signal cooperates with the CD40L-CD40 signal in playing a critical role in mTEC development and the establishment of self-tolerance of T cells in the thymus.

Mice lacking the *Tnfrsf11a* or *Tnfsf11* gene lack lymph nodes. The development of the secondary lymphoid organs is initiated during embryogenesis by the recruitment of lymphoid tissue inducer (LTi) cells to the lymphoid organ primordium.[89] This recruitment process is especially controlled by the chemokines produced by the anlage-resident stromal cell, lymphoid tissue organizer (LTo) cells. Both LTi and LTo cells express RANKL and RANK in the lymph node anlage during embryogenesis, suggesting that RANKL/RANK signaling is required for the interplay between LTi and LTo cells. Nevertheless, the formation of GALT, such as isolated lymphoid follicles and Peyer's patches are undisturbed in RANKL or RANK-deficient mice.[23,90] TNF receptor-associated factor (TRAF) 6, a crucial signal transducer of RANK signaling, is required for the development of lymph node development, but not for that of GALT,[90] indicating tissue-specific requirements of RANKL/RANK/TRAF6 signaling for lymph node organogenesis.

The microfold cell (M cell), located in the follicle-associated epithelium of GALT, is a kind of epithelial cell specialized for antigen sampling and important for the induction of efficient immune responses to mucosal antigens.[91,92] M cell differentiation requires the RANKL signal,[93] but the major source of RANKL in the gut remained unknown. Recently, a distinct type of subepithelial mesenchymal cells that highly expresses RANKL has been identified as an essential inducer for M cell differentiation [called M cell inducer (MCi) cells], which maintains the diversification of the gut microbiota by controlling IgA production.[94] Intestinal epithelium-specific deletion of the *Tnfrsf11a* gene results in the absence of the M cell, leading to defects of germinal center maturation in Peyer's patches and IgA production.[95] Thus, the RANKL/RANK signal is also important for the humoral acquired immune response in mucosal tissues.

As described earlier, RANKL was originally identified as a T-cell cytokine regulating DC function. Indeed, DC survival is supported by the TNF receptor superfamily members, such as RANK, CD40, and LTβR, that are expressed on DCs.[8,9,96] However, RANKL has no capacity to induce the expression of MHC class II and CD80/86 in DCs. Mice lacking the *Tnfrsf11a* or *Tnfsf11* gene exhibit no apparent defects in DC number and function,[97] suggesting that the CD40L/CD40 axis compensates for the loss of RANKL signaling. In support of this view, inhibition of RANKL signaling by treatment with the RANK-Fc fusion protein potently prevented T cell-dependent immune responses against viruses and parasites in CD40-deicient mice. Furthermore, a high expression of RANKL in T cells enhanced the survival of colonic $CD11c^+$ DCs in *Il2*-deficent mice, resulting in bone loss and a spontaneous form of colitis.[98] Adoptive transfer of RANKL-stimulated DCs exacerbated autoimmunity in MRL/lpr recipients.[99] Thus, the proinflammatory effect of RANKL on DCs was especially emphasized under certain experimental conditions. On the other hand, immunosuppressive roles of RANKL were also reported. RANKL on dermal keratinocytes has the ability to inhibit experimental autoimmune and allergic responses by increasing the number of Treg cells via epidermal DCs.[100] Moreover, administration of a neutralizing antibody against RANKL inhibits Treg-mediated suppression of colitis.[101] Inhibition of RANKL was shown to result in a rapid progression to diabetes due to a decrease in the Treg cell number.[102] However, there are no reports of severe immunodeficiencies in patients treated with the anti-RANKL antibody denosumab.[103] Again, it is possible that functional redundancy with the CD40 signal compensates for the defect of the RANKL/RANK signal in DCs.

In addition to DC regulation, the RANKL on T cells has been highlighted especially as a pathogenic effector of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. T cell-specific inactivation of RANKL inhibited infiltration of T cells into the central nervous system (CNS) and the development of EAE. The RANKL on T cells stimulates the production of CCL20 by astrocytes that express RANK, leading to the infiltration of T cells into the CNS. Interestingly, studies using single-cell transcriptomics demonstrated that RANKL is one of the key signatures of pathogenic effector T cells infiltrated into the CNS during EAE.[104] As pharmacological inhibition of RANKL prevented the onset and progression of EAE, RANKL inhibition might be a promising therapeutic strategy for multiple sclerosis.[105]

B cell–development was impaired in mice lacking the *Tnfrsf11a* or *Tnfsf11* gene. Also, in humans, *TNFSF11A*

mutations result in low levels of immunoglobulin-secreting B cells. Inhibition of the systemic RANKL/RANK system clearly leads to loss of the microenvironment for B cell–development due to severe osteopetrosis. The impaired development of bone marrow B cells was also observed in *Rag1*-deficient mice reconstituted with fetal liver cells from *Tnfsf11*-deficient mice, indicating that the RANKL expressed by hematopoietic lineage cells is essential for B cell–development. The number of pre-B cells was moderately but significantly decreased in B cell-specific RNAKL-deficient mice, indicating that the RANKL on B cells is involved in B cell–development. On the other hand, B cell-specific deletion of the *Tnfrsf11a* gene had no obvious effects on B cell–development, secretion of immunoglobulins, class switch recombination, or somatic hypermutation.[106] Further studies are needed to elucidate the detailed mechanisms by which the RANKL on hematopoietic cells regulates B cell–development.

4 INTRACELLULAR SIGNALING FOR OSTEOCLASTOGENESIS

Osteoclasts are large, multinucleated cells formed by the fusion of precursor cells of monocyte/macrophage lineage that originate from HSCs. Mature osteoclasts decalcify the inorganic components of bone by releasing hydrochloric acid, and degrade bone matrix proteins by secreting proteolytic enzymes, such as cathepsin K and MMPs. Studies on osteoclast biology have made a critical contribution to the development of osteoimmunology by providing information on the shared molecular mechanisms and interaction between immune and bone cells.

Before the identification of RANKL as an ODF, studies on naturally occurring or genetically modified mutant mice exhibiting osteopetrosis provided important clues for understanding the molecular mechanisms underlying osteoclast differentiation and function. For example, *op/op*, *gl/gl*, *oc/oc*, and *mi/mi* mice, which respectively carry a spontaneous mutation in the *Csf1*, *Ostm1*, *Mitf*, and *Tcirg1* genes, all develop osteopetrosis. Genetically modified mice deficient in PU.1, c-Fos, TRAF6, NF-κB p50/p52, c-Src, or TRAP also develop osteopetrosis.[107] The cloning of RANKL enabled further investigation of the signaling pathways for osteoclast differentiation in a sophisticated culture system using recombinant RANKL and M-CSF.

4.1 RANKL Signaling for Osteoclast Differentiation

RANKL binds to a type I transmembrane protein on the monocyte/macrophage linage cells called RANK to initiate osteoclast differentiation. RANK has no intrinsic enzymatic domain in its cytoplasmic domain, but does contain three putative TRAF-binding domains. Although RANK interacts with TRAF2, TRAF5, and TRAF6,[108] TRAF6 plays an indispensable role in osteoclast differentiation.[39,90] The other TRAF family members except for TRAF1 have a single N-terminal RING finger domain and are implicated in the signaling pathway downstream of various receptor families, including the TNF receptor superfamily, the Toll like receptor/IL-1 receptor family, IL-17R, IL-25R, and the NOD-like pattern recognition receptors.[109] It was reported that, upon homooligomerization, TRAF6 interacts with a K63-specific E2 conjugating enzyme via the RING finger domain, and in turn mediates the attachment of K63-linked ubiquitin chains to TRAF6 itself (autoubiquitination).[110] The K63-linked ubiquitin chain serves as a docking platform for downstream mediators, TGFβ-activated kinase (TAK) 1-binding proteins (TABs), and mitogen-activated kinase kinase kinase (MAPKKK) TAK1. However, K63-linked autoubiquitination of TRAF6 is not essential for the activation of the cascades downstream of RANK.[111,112] Upon RANKL stimulation, TRAF6 forms a complex with RANK, TAB1, and TAB2 to activate TAK1, which in turn activates NF-κB and MAPKs, such as JNK.[113,114] The essential role of TAK1 in osteoclast differentiation has been demonstrated using mice deficient in TAK1 in osteoclast linage cells.[114,115]

The NF-κB family of transcription factors consists of five members: p50 (processed from p105), p52 (processed from p100), RelA, RelB, and c-Rel.[116,117] Activation of NF-κB depends on canonical and noncanonical pathways.[118] In the canonical pathway, the IKK complex comprised of IKKα, IKKβ, and NF-κB essential modulator (NEMO)/IKKγ induces the phosphorylation and subsequent degradation of IκBs, leading to activation of mainly p50:RelA and p50:c-Rel dimers. The noncanonical pathway is dependent on the activation of the IKKα homodimer activated by the upstream kinase NF-κB-inducing kinase (NIK). IKKα phosphorylates p100 and induces the processing to p52, resulting in activation of the p52:RelB dimer. Mice doubly deficient in p50 and p52 develop severe osteopetrosis due to a lack of osteoclasts, whereas mice lacking either p50 or p52 exhibit no obvious bone phenotype,[119,120] indicating the importance of both the canonical and noncanonical pathways. The disruption of the *Ikkb* or *Rela* gene causes an impairment of osteoclast differentiation. In addition, loss of NIK blocks osteoclast differentiation by inhibiting the nuclear translocation of not only RelB, but also RelA. NEMO positively regulates osteoclast differentiation by suppressing the expression of a negative regulator for the differentiation, recombinant recognition sequence binding protein at the Jκ site (RBP-J).[121] Both the canonical and noncanonical pathways are necessary for RANKL-mediated osteoclastogenesis, but further studies

are needed to obtain a complete understanding of the complex mechanisms underlying the NF-κB activation downstream of RANK.

The important roles of the MAPK pathways downstream of RANKL have been demonstrated in a variety of in vitro experiments. Pharmacological inhibition of p38α and β by SB203580 blocks osteoclast differentiation of the RAW264.7 macrophage cell line.[122] In addition, the cells lacking p38α, the main p38 isoform in osteoclast linages, poorly differentiated into osteoclasts.[123] JNK1 is activated by RANKL and plays a critical role in osteoclast differentiation.[124] ERK1/2 are also activated by RANKL, but the role of ERK1/2 in osteoclast differentiation is controversial. Inhibition of MEK by U0126 and PD98059 promoted osteoclast differentiation in RAW264.7 cells,[125] whereas MEK inhibitor AZD6244 suppressed osteoclast differentiation.[126] Detailed analyses with in vivo models are needed for further understanding of the role of MAPKs in osteoclastogenesis.

The Fos family proteins (c-Fos, FosB, Fra-1, and Fra-2) form the AP-1 transcription complex together with Jun family proteins (c-Jun, JunB, and JunD). The critical role of c-Fos in osteoclastogenesis has been demonstrated by the findings that *cfos*-deficient mice develop osteopetrosis due to a lack of osteoclasts.[127–129] Downstream of RANK, c-Fos expression is induced by transcription regulators, such as the CaMK/cAMP responsive element binding (CREB) protein pathway,[130] NF-κB,[131] peroxisome proliferator activated receptor γ (PPARγ),[132] PPARγ coactivator 1 β (PGC1β),[133,134] and CCAAT/enhancer binding protein α (C/EBPα).[135] Fra-1 compensates for the loss of c-Fos, but *Fra1*-deficient mice not develop osteopetrosis. The abilities of FosB and Fra-2 to rescue the osteoclast differentiation blockade of *cfos*-deficient cells are relatively low. Thus, Fra-1, FosB, and Fra-2 do not exclusively function in the AP-1 signaling pathway in osteoclasts. Activation of c-Jun is also required for osteoclast differentiation.[124] Expression of dominant negative c-Jun in osteoclasts inhibited osteoclast differentiation in vivo. However, since dominant negative c-Jun represses the transcriptional activities of all AP-1 family members, there is the possibility of the involvement of other Jun family molecules in osteoclast development and function.[136] Indeed, studies using conditional knockout mice of JunB indicated the crucial role of JunB in osteoclasts.[137] Although the precise composition of the AP-1 dimers has not been determined in the physiological context, the AP-1 dimer composed of c-Fos and Jun proteins plays critical roles in osteoclast differentiation.

4.2 NFATc1, the Master Transcription Factor of Osteoclast Differentiation

Considering the fact that AP-1 and NF-κB transcription factors are also activated by various cytokines, such as TNF-α and IL-1, other transcription factors have been considered to be specifically involved in RANKL-mediated osteoclast differentiation. Based on the transcriptome analyses using osteoclasts, NFATc1 was identified as a transcription factor highly induced by RANKL and is now well-known to be the master transcription factor of osteoclast differentiation[138] (Fig. 16.2).

RANKL specifically and potently induces NFATc1 expression via TRAF6, c-Fos,[138] and p50/p52 NF-κB.[131] After induction, NFATc1 autoregulates its own promoter and thus enables the robust induction of NFATc1 itself in cooperation with other transcription factors, such as AP-1. NFATc1 is essential for osteoclast differentiation both in vitro[138] and in vivo,[139–141] and the overexpression of NFATc1 induces osteoclast differentiation without RANKL stimulation.[138] It has been shown that transcription factors, such as activating transcription factor 4 (ATF4),[142] B lymphocyte-induced maturation protein 1 (Blimp1),[143,144] leukemia/lymphoma-related factor (LRF),[145] and Jun dimerization protein (Jdp)2[146] negatively regulate NFATc1 induction.

NFATc1 directly controls the expression of various genes critical for osteoclast differentiation and function in cooperation with other transcription factors. Integrin β3, which is essential for both bone attachment and bone-resorbing activity,[147] is induced by NFATc1.[148] TRAP, a lysosome enzyme required for bone development and homeostasis,[149] is regulated by NFATc1 together with AP-1.[150] Cathepsin K, a member of the cysteine proteases important for osteoclastic bone resorption,[151] and immunoglobulin-like receptor osteoclast-associated receptor (OSCAR) are synergistically regulated by NFATc1, PU.1, and MITF.[152–154] Dendritic cell–specific transmembrane protein (DC-STAMP),[155] osteoclast stimulatory transmembrane protein (OC-STAMP)[156] and v-type proton ATPase subunit d2 (encoded by the *Atp6v0d2* gene),[157] which induce the fusion of osteoclast precursor cells, are controlled by NFATc1.[156,158] The expression of DC-STAMP is also regulated by c-Fos[159] and the transcriptional repressor strawberry notch homologue 2 (Sbno2).[160] Furthermore, posttranslational histone modifications, such as acetylation and methylation, are critical for the regulation of gene expression in osteoclasts. Histone deacetylation regulates the expression of c-Fos, NFATc1, and osteoclast-specific genes, such as cathepsin K during osteoclastogenesis.[161,162] Histone deacetylase (HDAC) 3 positively regulates osteoclast differentiation, whereas HDAC7 exerts a suppressive effect. Genome-wide ChIP-seq analysis revealed that the trimethylation of histone H3 lysine 27 (H3K27me3), which is a transcription marker of inactivity, occurs at the transcription start site of the *Nfactc1* gene in osteoclast precursor cells, but is eliminated by RANKL stimulation, suggesting that the H3K27me3 modification is important for NFATc1 induction during osteoclastogenesis.

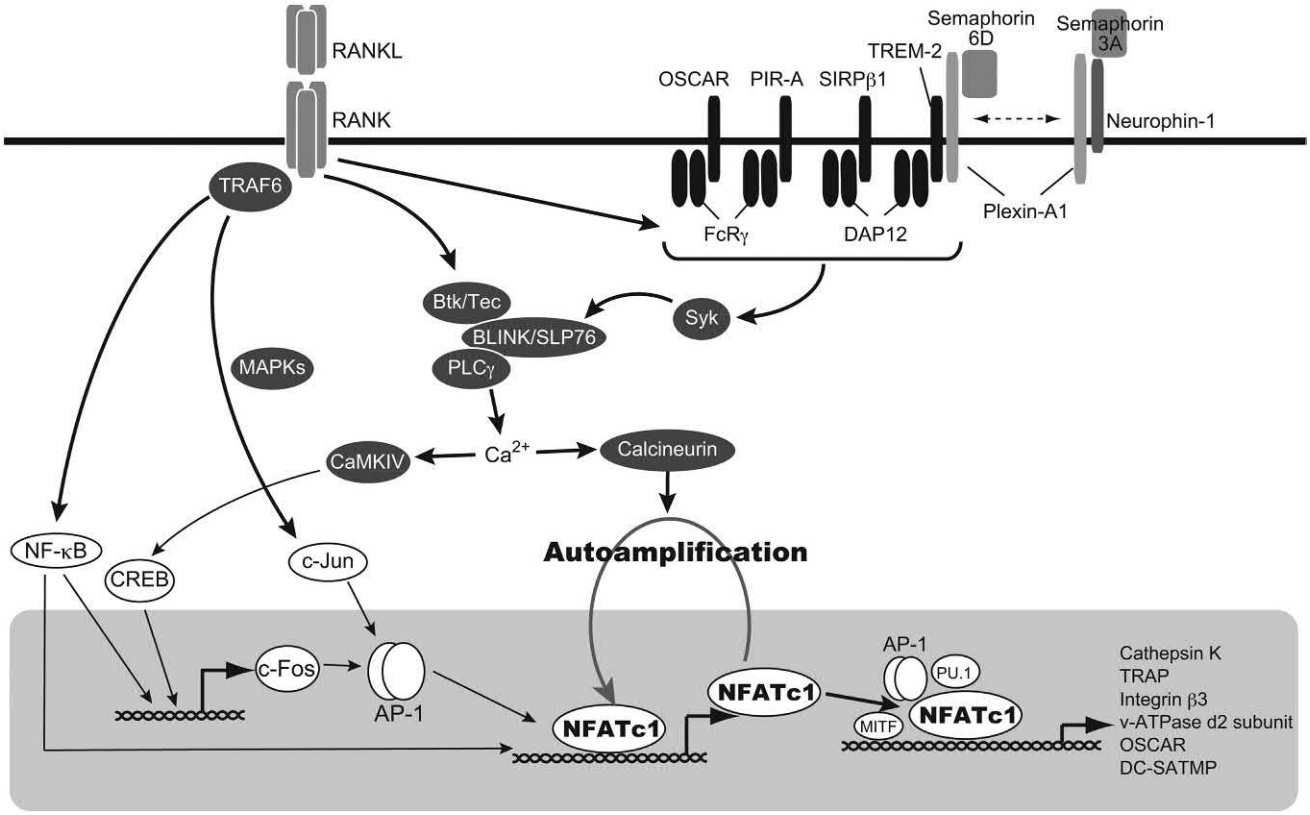

FIGURE 16.2 **NFATc1-centered osteoclastogenic signaling network.** RANKL binds to RANK on osteoclast precursor cells, leading to the activation of certain signaling pathways through TRAF6, including MAPK and NF-κB. RANKL also activates the ITAM signaling mediated by immunoglobulin-like receptors. The NF-κB pathway contributes to the induction and activation of c-Fos, and subsequent NFATc1 induction. The NFATc1 activated by Ca^{2+} signaling regulates the expression of osteoclast-specific genes.

Jumonji domain-containing 3 (Jmjd3), a histone H3K27-specific demethylase, plays a role in regulating NFATc1 expression through the demethylation of H3K27 in osteoclasts.[163]

4.3 Costimulatory Signal for RANK

The nuclear translocation and activation of NFATc1 is promoted by the dephosphorylation of NFATc1 by the Ca^{2+}/calmodulin-dependent phosphatase calcineurin. Calcineurin inhibitors, such as cyclosporine and tacrolimus potently inhibit osteoclast differentiation by suppressing NFATc1.[138] In osteoclast linages, NFATc1 activation is absolutely dependent on the calcium–calcineurin pathway induced by costimulatory signals from immunoglobulin-like receptors, such as OSCAR and triggering receptor expressed in myeloid cells-2 (TREM-2). These receptors associate with the adaptor molecules harboring an immunoreceptor tyrosine-based activation motif (ITAM): Fc receptor common γ subunit (FcRγ) and DNAX-activating protein 12 (DAP12; also known as Tyrobp).[164] Mice deficient in FcRγ do not exhibit any osteoclast defect in vivo or in vitro.[165] Mice deficient in DAP12 also have a normal number of osteoclasts but display mild osteopetrosis,[166] suggesting a role of DAP12 in the bone-resorbing activity of osteoclasts. However, mice doubly deficient in FcRγ and DAP12 exhibit severe osteopetrosis due to a complete lack of osteoclasts.[165] RANKL-induced calcium oscillation and NFATc1 expression are completely abrogated in cells lacking FcRγ and DAP12, indicating that the immunoglobulin-like receptor signal is essential for the activation of the calcium–calcineurin-NFATc1 pathway. Since FcRγ and DAP12 are well known molecules expressed mainly in myeloid cells and NK cells, these findings have had a serious impact on the fields of both immunology and bone biology. In osteoclasts, FcRγ associates with OSCAR and PIR-A, and DAP12 associates with TREM-2, SIRPβ, Myeloid DAP12-associating lectin (MDL)-1 and Siglec-15, respectively[165,167,168] (Fig. 16.2).

Fc receptors bind to the Fc domain of immunoglobulin or immune complexes. FcγRI, FcγRIII, and FcγRV are activating Fc receptors and associated with FcRγ, whereas FcγRIIB is an inhibitory receptor in mice. Among these receptors, FcγRIIB and FcγRIII are highly expressed in osteoclast progenitor cells.[169] FcγRIIB and FcγRIII inhibit osteoclastogenesis by suppressing ITAM signals and sequestering FcRγ from OSCAR and PIR-A,

respectively. However, in autoimmune diseases, such as RA, the immune complex enhances osteoclastogenesis by activating FcγRIII, indicating that this immune complex directly regulates bone metabolism through the regulation of osteoclasts (see Section 5).

Once phosphorylated, the ITAM-containing adaptors recruit spleen tyrosine kinase (Syk). Syk is required for ITAM signaling in osteoclast differentiation because *Syk*-deficient cells fail to differentiate into osteoclasts in response to RANKL.[170] In various immune cells, such as lymphoid and myeloid cells, Tec tyrosine kinase family proteins are well-known targets of Syk[171,172] and regulate its function. ITAM-mediated T- and B-cell receptor signaling is controlled by Itk and Rlk,[173] and Btk and Tec,[174] respectively. In osteoclast linage cells, Btk and Tec are highly expressed and function as essential kinases for ITAM-mediated signaling. Mice deficient in both Btk and Tec develop severe osteopetrosis owing to a lack of osteoclasts.[175] The Tec and Btk activated by RANK cooperate with Syk and SH2-containing leukocyte protein (SLP) family proteins, B-cell linker protein (BLNK) and lymphocyte cytosolic protein 2 (Lcp2; also known as SH2-containing leukocyte protein of 76 kDa, SLP76), to induce efficient phosphorylation of phospholipase Cγ (PLCγ).[175] PLCγ hydrolyzes the phospholipid, phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2) to generate the second messengers diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3).[176,177] IP_3 provokes a transient increase in the intracellular calcium released from the endoplasmic reticulum (ER) Ca^{2+} store through IP_3 receptors (IP_3Rs) at the ER membrane.[178] IP_3R2 and IP_3R3 are mainly expressed in osteoclast linages. Although cells lacking both of the IP_3Rs fail to differentiate into osteoclasts in vitro due to an impairment of the calcium oscillation,[179] mice deficient in IP_3R2 and IP_3R3 do not show any bone abnormality, suggesting that the loss of IP_3R2 and IP_3R3 is compensated by other, as yet unknown mechanisms in vivo. Calcium oscillation is induced and strictly regulated by both the increased intracellular Ca^{2+} and removal of intracellular Ca^{2+} from the cytosol. During osteoclastogenesis, this process is regulated by the sarco/ER Ca^{2+} ATPase (SERCA) 2 that reuptakes Ca^{2+} into the intracellular Ca^{2+} store,[180] the transmembrane protein 64 (Tmem64) that associates with SERCA2,[181] and regulator of G-protein signaling 10 (RGS10).[182] In summary, the immunoglobulin-like receptor and ITAM-harboring molecules induce costimulatory signals critical for PLCγ activation, RANKL-induced calcium oscillation, and NFATc1 activation (Fig. 16.2).

4.4 Negative Regulators of Osteoclastogenic Signaling

Various negative regulatory molecules are known to exert an effect on inactivating the RANKL signaling pathway in osteoclasts. Mice with a mutation in the *Ptpn6* gene encoding SH2-domain-containing phosphatase 1 (SHP-1), a nonreceptor type of tyrosine protein phosphatase, display a low bone mass due to an increased number of osteoclasts.[183,184] SHP-1 negatively regulates osteoclastogenesis by suppressing the PI3K-Akt pathway[185] and associating with inhibitory Ig-like receptors, such as PIR-B.[186] The inositol phosphatases SH2-domain-containing inositol 5′-phosphatase 1 (SHIP1) and phosphatase and tensin homolog (PTEN) act as negative regulators of osteoclast differentiation.[187,188] In addition, the transcriptional factors interferon regulatory factor-8 (IRF-8)[189] and B cell lymphoma 6 (Bcl6)[143] also contribute to the negative regulation of osteoclastogenesis by directly interacting with NFATc1 and suppressing NFATc1 expression, respectively. In response to RANKL, the expression of IRF-8 and Bcl6 is downregulated through Blimp1.[143,144] The downregulation of IRF-8 is associated with DNA methylation of the *Irf8* gene by the DNA methyltransferase 3A (Dnmt3a).[190] A gain-of-function mutation in the *Sh3bp2* gene causes cherubism characterized by inflammation and enhanced bone resorption in the face. Mice carrying the cherubism mutation in the *Sh3bp2* gene exhibit severe osteoporosis with enhanced bone resorption.[191] In mature osteoclasts, SH3BP2 regulates bone-resorbing activity through the Src and Vav pathways.[192] MicroRNA is known to suppress the expression of its target genes at the translation level. One of the microRNAs, miR-34a, has been shown to inhibit osteoclast differentiation by suppressing the expression of transforming growth factor-β-induced factor 2 (Tgif2), which regulates JNK and NF-κB signals downstream of RANK.[193] Recently, leucine-rich repeat-containing G-protein-coupled receptor 4 (LGR4) was reported to be another receptor for RANKL.[194] LGR4 negatively regulates osteoclastogenesis by inhibiting NFATc1 activation via Gαq, as well as competing with RANK for RANKL binding. This finding may have a profound influence on RANKL biology, but further studies will be needed to test the importance of LGR4-mediated regulation of RANKL under both physiological and pathological conditions.

Osteoclast lineage-specific conditional knockout mice generated by the Cre-loxP system have provided important insights into the essential molecules for osteoclasts, contributing to outstanding advances in the field of osteoclast biology. However, it is important to carefully check the deletion efficiency and the timing of Cre-mediated recombination, since the deletion efficiency depends on the compatibility with flox mice. For example, deletion of the *Nfatc1* gene in NFATc1 flox mice crossed with *LysM-Cre* mice was not observed in bone marrow macrophages and the mice did not exhibit any bone phenotype.[141] Thus, the deletion efficiency and the

protein expression level of the gene need to be carefully evaluated in the osteoclast linages.

4.5 Cell–Cell Communication Between Osteoclasts and Osteoblasts

The entire course of the bone remodeling process is achieved by the interplay among osteoclasts, osteoblasts, and osteocytes, in cooperation with other cells in the bone marrow, not least immune cells. The molecules that function in the cell–cell interaction in bone remodeling have come to be referred to as "communication factors." RANKL is thus the prototypical communication factors, but other molecules have been recently identified as additional communication factors controlling osteoclastogenesis.

The axon guidance molecules the Ephrins and their Eph receptors transduce bidirectional signals through the receptor (forward signaling) and through the ligand (reverse signaling) in contact-dependent cell–cell communication.[195] EphrinB2 and its receptor EphB4 are expressed on osteoclasts and osteoblasts, respectively.[196] The forward signal through EphB4 promotes osteoblast differentiation by activating the small GTPase RhoA, whereas the reverse signal through EphrinB2 suppresses osteoclast differentiation by inhibiting c-Fos and NFATc1.[196] The EphrinB2/EphB4 signal may allow transition from bone resorption to new bone formation by terminating bone resorption and simultaneously inducing bone-formation activity. The Semaphorin family of proteins, which were originally identified as axonal growth cone guidance molecules, regulates the immunoglobulin-like receptor signal. Sema6D, which is a ligand for Plexin-A1 (PlxnA1), stimulates osteoclast differentiation by activating the ITAM signal through the formation of the PlxnA1-TREM2-DAP12 complex.[197] The Sema3A produced by osteoblasts binds to the receptor neuropilin1 (Nrp1) in osteoclast precursor cells and inhibits their differentiation by blocking PlxnA1-TREM2-DAP12 complex formation.[198] By binding to Nrp1/PlxnA1 complex on osteoblast lineage cells, Sema3A also stimulates osteoblast differentiation through activation of the canonical Wnt/β-catenin pathway.[198] Thus, Sema3A exerts an osteoprotective effect by both promoting bone formation and simultaneously inhibiting bone resorption. It was also reported that sensory nerve innervation into bone by neuron-derived Sema3A is involved in bone remodeling during development.[199] Osteoblast-derived Wnt16 suppresses osteoclastogenesis by both inducing OPG in osteoblasts and directly acting on osteoclasts through a noncanonical Wnt signal.[200] On the other hand, osteoblast-derived Wnt5a binds to Ror2 on osteoclast precursor cells and upregulates RANK expression, thereby enhancing RANKL-induced osteoclast differentiation.[201] In addition to these factors, various soluble factors secreted from osteoclasts were reported to act as communication factors regulating osteoblastc bone formation, including Sema4D,[202] leukemia inhibitory factor,[203] CTHRC-1,[204] and PDGF-BB.[205,206] Interestingly, the PDGF-BB from preosteoclasts also regulates angiogenesis in the bone marrow and periosteum to further support bone formation.[206]

5 MECHANISMS UNDERLYING BONE DESTRUCTION IN ARTHRITIS

RA is an autoimmune disease characterized by chronic inflammation of the synovium and subsequent bone destruction in multiple joints.[207] RA is the most representative skeletal disorder directly triggered by an abnormal or/and prolonged activation of the immune system. Although genetic susceptibility and environmental triggers are both reportedly involved in the etiology of RA, the exact cause remains uncertain. To develop effective therapeutic strategies against RA, attention must be paid to how abnormality of the immune system, especially autoreactive T lymphocytes, ultimately leads to the bone destruction. Thus, to explore the mechanism underlying bone destruction associated with RA has been one of the driving motives of osteoimmunology.

In the early 1980s, osteoclast-like cells was identified in the inflamed synovium of rheumatoid joints,[208] but it was not until RANKL was cloned that the importance of osteoclasts in arthritis attracted much attention. The findings that osteoclast formation was consistently observed in synovial cell cultures from patients with RA clearly indicated that the inflamed synovial tissues contain both osteoclast precursor cells and osteoclastogenesis-supporting cells.[209] After RANKL was cloned as a factor essential for osteoclastogenesis, it turned out that RANKL expression was also detected specifically in the synovium of patients with RA.[210,211] Furthermore, the importance of RANKL and osteoclasts in the bone destruction associated with inflammation was demonstrated in mouse models of autoimmune arthritis.[212,213] Mice lacking osteoclasts or osteoclast activity were protected from bone destruction, but not inflammation, in arthritis models. Anti-RANKL and antiosteoclast therapies were shown to exert beneficial effects in treating bone loss in animal models of arthritis.[214,215] In a RA patient with osteopetrosis, bone erosion was hardly observed despite severe inflammation and cartilage destruction.[216] Taken together, these genetic findings demonstrate the indispensable role of osteoclasts in the bone destruction that occurs in RA.

Inflammatory cytokines produced in the inflamed synovial tissues, including TNF-α, IL-6, and IL-1, directly induce RANKL expression on osteoclastogenesis-supporting cells, such as synovial fibroblasts and facilitate

RANKL signaling. RANKL is abundantly expressed in synovial fibroblasts in the inflamed synovium, but has also been shown to be expressed on T and B cells.[6,211,214,217,218] There has been a long-standing debate over the source of RANKL in arthritis. Soon after the cloning of RANKL, in vitro culture experiments showed that fixed activated T cells act directly on osteoclast precursor cells and induce osteoclastogenesis via the RANKL on T cells.[214] However, living T cells do not possess such an osteoclastogenic capacity as they produce cytokines, such as IFN-γ, which exert potent inhibitory effects on osteoclast differentiation.[6] As we will discuss later, one of the helper T cell subsets, Th17 cells, functions as an osteoclastogenic T cell subset, expressing a higher level of RANKL than any other T cell and inducing RANKL expression on mesenchymal cells, such as synovial fibroblasts.[12] Studies using RANKL conditional knockout mice showed that the bone destruction and osteoclast formation in the joints was inhibited in the mice, in which the *Tnfsf11* gene had been deleted in joint mesenchymal cells, including synovial fibroblasts, using *Col6a-Cre* mice. On the other hand, T cell-specific deletion of the *Tnfsf11* gene had no effect on bone destruction. Thus, synovial fibroblasts, not T cells, are functionally most relevant as a source of RANKL in arthritic joints.[219]

5.1 Th17 Cells and Treg Cells in Autoimmune Arthritis

Accumulating evidence lends support to the view that $CD4^+$ T cells play a key role in the pathogenesis of RA. Infiltration of $CD4^+$ T cells into the synovium of the affected joints and the presence of autoantibodies are pathological hallmarks of RA. Genome-wide association studies revealed *PTPN22* and *CCR6*, which are related to TCR signaling and $CD4^+$ T cell migration, respectively, as well as HLA-DRB1 alleles as the most relevant in RA susceptibility.[220–224] CTLA-4-Ig (abatacept), a selective inhibitor of T cell activation, was shown to be effective against RA.[225] Moreover, T cell-depletion protected mice against the development of CIA and KBxN arthritis, which are animal models of RA.[226,227] Thus, considering the primary role of $CD4^+$ T cells in the inflammation and bone destruction of RA, attention must be focused to how T-cell immunity is linked to osteoclast-mediated bone destruction.

Upon TCR activation, naive $CD4^+$ T cells differentiate into different lineages of helper T (Th) cells, depending on the cytokine milieu; Th1, Th2, Th17, inducible Treg (iTreg), and T follicular helper (Tfh) cell subsets. These Th cell subsets are defined by their pattern of cytokine production and immune function. Th1 and Th2 cells are traditionally thought to be the major subsets generated upon antigenic stimulation. Th1 cells, which are induced by IL-12, produce mainly IFN-γ and are critical for host defense against intracellular viral and bacterial pathogens. Th2 cells produce mainly IL-4, IL-5, and IL-13 and contribute to allergic responses and the parasite defense system. Th17 cells, which were discovered in 2000s as an IL-17-producing distinct Th cell subset, confer protection against extracellular bacterial and fungal pathogens, and importantly, contribute to autoimmune inflammation. Activated T cells express not only RANKL but also effector cytokines with either stimulatory or inhibitory effects on osteoclastogenesis.[6,7] Th1 and Th2 cells strongly inhibit osteoclastogenesis by producing IFN-γ and IL-4, respectively. In contrast, Th17 cells mainly potentiate osteoclastogenic activity by producing IL-17, which has the capacity to induce RANKL on osteoclast-supporting mesenchymal cells.[12] Under arthritic conditions, IL-17 produced by Th17 cells directly acts on arthritic synovial fibroblasts and stimulates innate immune cells to produce inflammatory cytokines, such as TNF-α, IL-1β, and IL-6. These cytokines further promote osteoclastogenesis by upregulating RANKL on synovial fibroblasts and directly activating osteoclast precursor cells, resulting in severe bone destruction (Fig. 16.3). Synovial cells stimulated by inflammatory cytokines also produce matrix-degrading enzymes that cause cartilage destruction. Thus, Th17 cells play a central role as the pathogenic link between the inflammatory response and joint destruction in RA. Furthermore, the Th17–synovial fibroblast interaction is deeply associated with RA pathogenesis. Under arthritic conditions, synovial fibroblasts promote the migration of Th17 cells to the inflammatory joint via CCL20, and proliferation of Th17 cells and IL-17 production via IL-6.[228–230] The interaction between the immune cells and synovial fibroblasts may hold the key to understand the joint specificity of RA pathogenesis.

Treg cells are an immunosuppressive $CD4^+$ T cell subset that engages in the maintenance of immunological self-tolerance and immune homeostasis. Their physiological significance has been evidenced by the development of severe autoimmune disease, allergy, and immunopathology in humans and mice with a mutation of forkhead box P3 (Foxp3), a master regulator for Treg cell.[231] In contrast to Th17 cells, Treg cells are reported to have inhibitory effects on RANKL-induced osteoclastogenesis, but no consensus about the mechanism of action has been reached. Some studies have reported that Treg cells suppress osteoclast differentiation via TGF-β and IL-4[232] or TGF-β and IL-10,[233] while others reported that their suppressive effect is cell–cell contact dependent via CTLA-4.[234] By binding to CD80/86 on osteoclast precursor, CTLA-4 induces activation of the enzyme indoleamine 2,3-dioxygenase (IDO), which promotes apoptosis.[235] Using in vivo analyses, Treg cells have been shown to protect against local and systemic bone loss in a mouse model of TNF-α-induced arthritis.[236] In addition, Foxp3 transgenic mice display

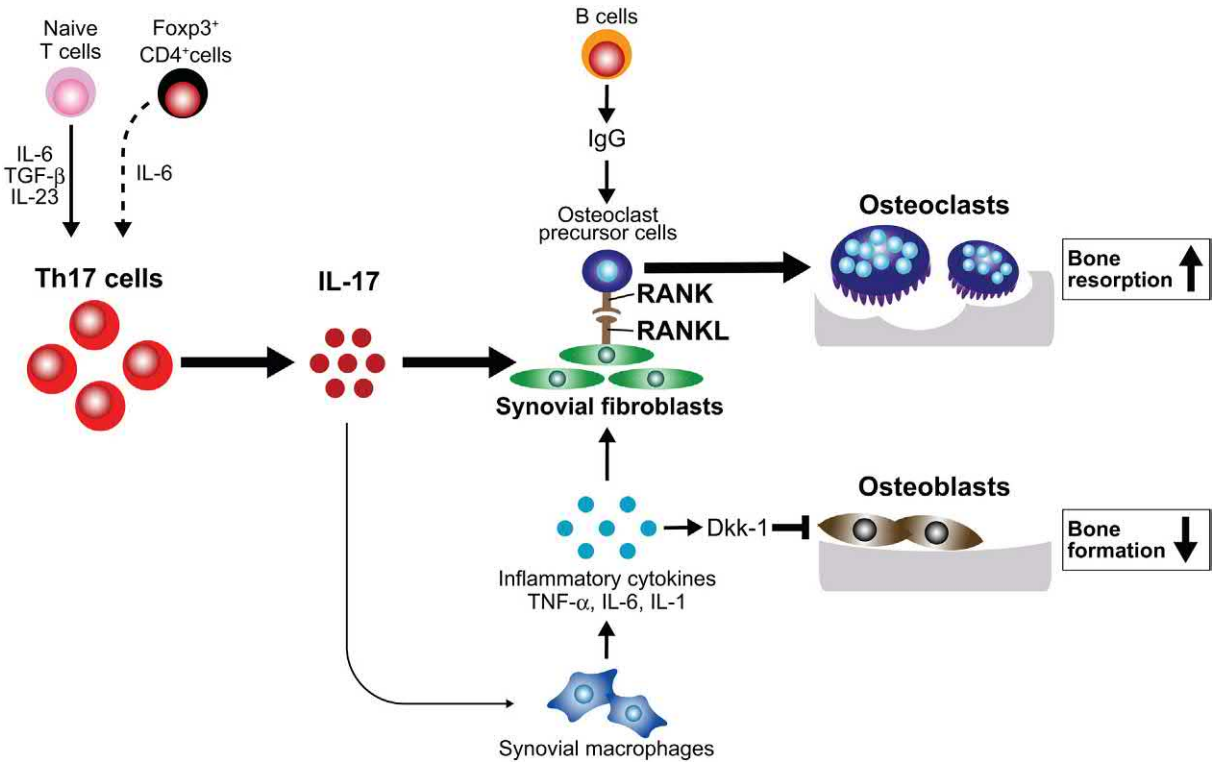

FIGURE 16.3 **Bone destruction in rheumatoid arthritis.** The bone destruction in arthritis occurs in the inflammed synovium at the interface of the immune system and bone. IL-17 produced by Th17 cells upregulates RANKL expression on synovial fibroblasts and induces inflammatory cytokines, such as TNF-α, IL-6, and IL-1 from synovial macrophages. These cytokines further upregulate RANKL expression on synovial fibroblasts and activate osteoclast precursor cells. The IgG immune complex directly promotes osteoclast differentiation. RANKL stimulates osteoclastic bone resorption, while the DKK-1 induced by inflammatory cytokines such TNF-α suppresses bone formation.

high bone mass and are partially resistant to ovariectomy (OVX)-induced bone loss.[237] Taken as a whole, it is likely that Treg cells serve as an inhibitory modulator in bone destruction in RA, but it is important to consider the possibility that the characteristics of Treg cells are affected by the specific inflammatory microenvironment. Although a number of Treg cells have actually been observed within RA patient joint fluid,[57–59] these Treg cells in the joint fluid displayed compromised immunosuppressive function. Studies using a mouse model of autoimmune arthritis demonstrated that CD-25^{lo}Foxp3^{+} T cells did lose Foxp3 expression under arthritic conditions and converted into a novel Th17 cell subset (termed exFoxp3Th17 cells) with the support of the IL-6 produced by arthritic synovial fibroblasts. Notably, in the presence of arthritic synovial fibroblasts, exFoxp3Th17 cells exert a greater capacity for inducing osteoclastogenesis than naïve CD4^{+} T-derived Th17 cells. Moreover, IL-17^{+}FOXP3^{+} T cells were observed in the synovium of RA patients with high disease activity, but not low disease activity, suggesting a contribution of Foxp3^{+} T cell plasticity to the pathogenesis of RA[238] (Fig. 16.3). The conversion of Foxp3^{+} T cells into effector T cells has been reported in diseases, such as diabetes, multiple sclerosis, and asthma, suggesting the importance of exFoxp3 T cells in the pathogenesis of autoimmune and allergic diseases.[239–241]

5.2 Therapeutic Strategies for RA

The inflammatory cytokines, such as TNF-α and IL-6 are responsible for not only local inflammation but also bone destruction through the upregulation of RANKL. The blockade of IL-6 or TNF-α has been shown to be efficacious treatment for RA. Even patients who were unresponsive to an anti-TNF-α (antibody) or anti-IL-6R antibody with regard to the inflammatory index still showed improvement of bone damage, suggesting direct inhibitory effects on osteoclastogenesis.[242,243] The effectiveness of CTLA4-Ig points to the relative contribution of T cell activation even in the bone destruction phase. Since CTLA-4 is also involved in osteoclast regulation as mentioned earlier, it is possible that CTLA4-Ig directly acts on osteoclastogenesis. An anti-RANKL antibody is widely used for the treatment of bone metastasis and osteoporosis and is being tested in clinical trial for bone destruction in RA.[244] An anti-IL-17A Ab has been shown to be less effective than the blockade of IL-6 or TNF-α in RA, despite the

well-established significance of Th17 cells in mouse models of RA.[245,246] It is possible that Th17-related cytokines other than IL-17A may be important for RA pathogenesis. Alternatively, considering that the pathogenesis of RA seems to be heterogeneous in patients, animal models of RA do not always represent all of the patients with the human form of the disease. An anti-IL-17A Ab has been shown to be very effective in psoriatic arthritis and ankylosing spondylitis.[247–249] Thus, it is important to deeply understand the difference in the mechanisms underlying the effects of T cells on bone tissues among RA, psoriatic arthritis, and ankylosing spondylitis. JAK inhibitors are effectively used for RA but the precise mechanisms, by which JAK inhibitors block bone damage, remain elusive.

Successful treatment of RA with B cell–depleting anti-CD20 monoclonal antibodies has highlighted the importance of B cell function in RA.[250,251] Autoantibodies against citrullinated proteins and IgGs are widely used to diagnose RA and form a prognosis for bone damage. Such autoantibodies have the capacity to enhance osteoclastogenesis in human and mouse culture systems.[169,252,253] The IgG immune complex directly regulates osteoclast differentiation via FcRγ receptors under arthritic conditions[169,253] (Fig. 16.3). Thus, the immune complex not only promotes inflammation, but also directly induces bone destruction in arthritis. Synovial fibroblasts produce Dickkopf-1 (DKK-1) in response to inflammatory cytokines, such as TNF-α, leading to inhibition of osteoblastic bone formation (Fig. 16.3). An anti-DKK-1 antibody enhances bone formation and ameliorates bone loss,[254] suggesting that upregulating bone formation may be a helpful strategy for the regeneration of eroded joints. The development of novel strategies for enhancing bone formation and inhibiting bone destruction at the same time is much anticipated in the treatment for RA.

The advances of osteoimmuonology has greatly contributed to the understanding of the mechanisms by which immune system abnormalities lead to the bone destruction in RA and thus provide the molecular basis for the development of the novel therapeutic strategies. The osteoimmunological viewpoint will be indispensable for a better understanding of the pathogenesis of other bone diseases associated with abnormal immune responses, such as periodontitis and ankylosing spondylitis.[255]

6 BONE MARROW MICROENVIRONMENT

HSCs have the capacity to differentiate into all immune cells and reside in microenvironments (niches) in the bone marrow in adults.[256] HSC activities are regulated by both intrinsic and extrinsic signals, the latter of which are from niche cells. Accumulating evidence has revealed that various cell types resident in the bone marrow, including bone cells, endothelial cells, neural cells, and stromal cells, play crucial roles in the regulation of immune cell development and function.[3] Osteoblasts were first reported to contribute as the HSC niche, which brought researchers to pay considerably more attention to the importance of osteoblasts in hematopoiesis. However, the mechanisms by which constituents of the bone marrow microenvironment support HSC activity are profoundly complex, so the true identity of the cells regulating HSCs is still incompletely understood. The role of bone cells in the maintenance of HSCs and immune cells in the bone marrow microenvironment has emerged as one of the most important subjects of osteoimmunology.

Osteoblasts continuously form new bone, providing a structural platform for the maintenance of HSCs in the bone marrow. It was initially proposed that the endosteal surface contains a key component for supporting HSCs.[257–259] In 2003, two independent groups reported the critical role of osteoblasts in HSC regulation in vivo by showing that the osteoblast number was associated with the HSC number using PTH/PTH-related protein receptor (PPR) transgenic and bone morphogenetic protein IA (BMPRIA) conditional knockout mice.[260,261] These pioneering studies highlighted the importance of osteoblasts for the regulation of HSCs. The findings that osteoblast ablation affected the HSC population[262] and that fetal skeletal progenitors have the capacity to reconstitute the ectopic HSC niche formation[263] further supported a close relationship between the osteoblasts and the HSCs in bone marrow. Osteoblasts were also reported to express key molecules for HSC regulation, including N-cadherin, angiopoietin-1, thrombopoietin, and osteopontin.[261,264–266] In contrast, other studies have reported that osteoblasts are less relevant to maintaining HSCs. Biglycan-deficient mice exhibit an osteoporotic phenotype but have a normal number of HSCs.[267] An increase in osteoblasts by strontium ranelate injection had no effect on the HSC number.[268] Furthermore, studies using conditional gene targeting in osteoblasts demonstrated that N-cadherin and Ang-1 on osteoblasts is dispensable for HSC maintenance.[269,270] Thus, osteoblasts may not be required for HSC maintenance under certain conditions.

Various cell types including CAR cells, leptin receptor-expressing ($LepR^+$) perivascular stromal cells, $Nestin^+$ perivascular cells and nonmyelinating Schwann cells are important for HSC maintenance.[271–274] The selective depletion of CAR cells in vivo reduced both the HSC and progenitor number. LepR-expressing perivascular stromal cells were shown to be a major source of stem cell factor in the bone marrow and to support HSCs.[271,275,276] In contrast, osteoblast lineage-specific deletion of stem cell factor or CXCL12 did not affect the HSC number. HSC niche cells thus far been characterized by different

research groups based on cytokine expression pattern, surface markers, and localization in the bone marrow. It will be necessary to integrate all such findings into a unified scheme to ultimately unveil the true nature of HSC niche cells, as well as their regulation.

The contribution of osteoblasts to HSC maintenance may not be as essential as suggested by earlier studies, but it has become clear that osteoblasts are involved in the modulation of immune cell differentiation in the bone marrow. Deletion of the *Cxcl12* gene in osterix-expressing mesenchymal cells, which are mostly osteoblasts, resulted in a decrease in B-lymphoid progenitors in the bone marrow.[262,277,278] A recent study showed that acute inflammation potently suppresses osteoblastic bone formation, resulting in a defect of lymphocyte development in the bone marrow. Osteoblasts play a role in the regulation of common lymphoid progenitors by providing IL-7.[279] The delta-like 4 (DLL4) on osteoblasts also contributes to supporting T cell competent progenitors in the bone marrow.[280] Conditional deletion on osteoblasts of RNase III endonuclease essential for microRNA biogenesis Dicer1 resulted in both impaired osteoblastic differentiation and myelodysplasia, the latter induced by a change in the bone marrow microenvironment,[281] indicating that osteoblast function alteration induced the perturbation of HSCs. The constitutive activation of β-catenin through FoxO1 in osteoblasts induced abnormal proliferation of neutrophils.[282] Taken together, osteoblasts regulate the fate of the immune cell progenitors by a variety of signals in the bone marrow.

Osteoclastic bone resorption is essential for the formation of the bone marrow cavity. Thus, mice lacking osteoclasts exhibit extensive extramedullary hematopoiesis in the spleen and liver because of insufficient space for support of hematopoietic cells within the bone marrow.[24,283] An aberrant microenvironment might provoke impaired immune cell differentiation and function.[284] Indeed, osteopetrotic patients develop anemia and serious infection due to abnormal hematopoiesis.[285–287] In *oc/oc* mice, which have inactive osteoclasts because of a mutation in the *Tcirg1* gene,[288] and mice treated with zoledronic acid, the frequency and absolute number of $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ (LSK) cells were decreased. The absence of osteoclast activity induces a severe alteration in endochondral ossification, resulting in a defective HSC niche.[289] It is also shown that inhibition of osteoclast activity by zoledronic acid impairs B cell–development by suppressing IL-7 and CXCL12 expression from stromal cells.[290] These results suggested that niche space formation by osteoclastic bone resorption is required for normal hematopoiesis.

HSCs are retained in the bone marrow because they express CXCR4, which is a receptor for CXCL12 on niche cells. There is a report showing that osteoclasts modulate the interaction between the CXCR4 on HSCs and CXCL12 on niche cells by secreting MMP 9.[291] Thus, HSC mobilization may be induced by the activation of osteoclasts. G-CSF is widely used as an HSC mobilizer in bone marrow transplantation,[292] but several reports have indicated the role of osteoclasts to be dispensable in such G-CSF-induced mobilization. G-CSF injection did not affect the osteoclast number on the endosteal surface. G-CSF-induced mobilization of HSCs is not impaired in osteoclast-deficient mice, such as *op/op*, $cfos^{-/-}$, and $Tnfsf11^{-/-}$ mice, or mice treated with zoledronic acid.[293,290] Instead, osteoblasts and osteocytes are required for the mobilization of HSCs in response to G-CSF.

When osteoclasts degrade the bone matrix, certain factors stored in the matrix are released. Near the sites of bone resorption, the local concentration of Ca^{2+} is considerably higher than in serum. HSCs lacking the calcium-sensing receptor fail to lodge at the endosteal sites near resorbing osteoclasts,[294] suggesting that the high Ca^{2+} level resulting from bone resorption is involved in HSC maintenance. TGF-β plays an important role in regulating HSC quiescence and self-renewal.[274] TGF-β is deposited in bone matrix in an inactive form that is activated by proteolytic enzymes secreted from osteoclasts. In this context, osteoclast activity may be involved in TGF-β-mediated HSC maintenance. Osteoclasts might contribute to HSC regulation by releasing the matrix-embedded factors that control HSCs.

Osteocytes, the most abundant cells in bone, are terminally differentiated osteoblast lineage cells embedded within mineralized bone matrix. Osteocyte-ablated mice exhibited defects in the bone marrow, thymus, and spleen, resulting in severe lymphopenia,[295] but the mechanisms by which osteocytes regulate lymphocyte development remain elusive. Sclerostin (encoded by the *Sost* gene), which is mainly expressed by osteocytes in bone, inhibits the Wnt-signaling pathway by binding to the LRP5/6 receptors in mesenchymal stromal cells.[296] As inhibition of the Wnt pathway leads to decreased bone formation, $Sost^{-/-}$ mice exhibit a high bone mass phenotype.[297] Notably, the number of mature B lymphocytes was reduced in the bone marrow of $Sost^{-/-}$ mice due to the reduced expression of CXCL12.[298] In addition, there is a report showing that osteocytes regulate the differentiation of myeloid linage cells. Osteocyte-specific Gsα-deficient mice have increased neutrophils and platelets due to upregulation of G-CSF.[299] Activation of the PTH receptor on osteoblasts induces an increase in the HSC number, as mentioned previously,[260] whereas activation of the PTH receptor on osteocytes did not affect the HSC number or function despite an increase in osteoblasts.[300] It is thus suggested that PTH signaling in osteocytes is insufficient to increase HSCs. Considering the number of osteocytes in bone tissues, they are suggested to affect the bone marrow microenvironment, but the role of osteocytes in hematopoiesis has been poorly elucidated to

date. More detailed studies will be needed to determine the function of osteocytes in immune cell differentiation in the bone marrow.

Early studies suggested an important role of osteoblasts in the HSC niches. This concept has been modified but nevertheless attracted the attention of various researchers to the role of bone cells in HSC maintenance. The role of osteoblast-lineage cells in hematopoiesis has been proven to be more limited than previously expected, but recent studies have demonstrated that osteoblasts are specifically important for the differentiation of certain immune cells, including lymphoid cells. New techniques of visualizing the bone marrow microenvironment and improvements in genetic manipulation will lead to further insights into the biological significance of bone cells in regulating immune cell development.

7 CONCLUSIONS

The bone is a multifunctional organ that both regulates itself and responds to a range of stimuli, including calcium intake, mechanostress, aging, and inflammation. As the bone carries out diverse functions related to the needs of the skeletal, endocrine, and immune system, studies on bone have been carried out by researchers from different disciplinary fields. However, integrating the knowledge from each discipline is necessary to understand the multifunctional aspects of bone. Osteoimmunology is a good example of such interdisciplinary unification, and has provided crucial insights needed for the development of novel therapeutic strategies in bone and joint diseases, as well as immune disorders. The findings of osteoimmunology from studies on bone destruction in RA will provide a critically important context for a better understanding of other skeletal diseases as well.

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CHAPTER

17

Integrating Endocrine and Paracrine Influences on Bone; Lessons From Parathyroid Hormone and Parathyroid Hormone-Related Protein

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1 BONE REMODELING AND MODELING

Bone formation and resorption proceed throughout life. When these processes cause changes in bone shape during skeletal growth, the term *modeling* is used. Modeling takes place from the beginning of skeletogenesis during fetal life until the end of the second decade when longitudinal growth of the skeleton is completed. In the modeling process, bone is formed at a location different from the sites of bone resorption, leading to a change in the shape, or macroarchitecture, of the bone. Modeling is responsible for determining the size and shape of bone, such as the simultaneous widening of long bones and expansion of the medullary cavity by coordinated bone formation at the periosteal surface with resorption at the endosteal surface. In contrast, *remodeling* is an adaptive process that occurs throughout life, whereby small packets of bone are resorbed, and then replaced asynchronously all over the skeleton at anatomically distinct sites termed basic multicellular units (BMUs). This process is an integral part of the calcium homeostatic system and provides a mechanism for repair and adaptation to physical stress. Remodeling thus maintains the mechanical integrity of the skeleton by replacing old with new bone, and repairing damaged bone.[1–4] Remodeling is most rapid early in life, and becomes slower from about the third decade in human subjects. It continues with age and in women accelerates over the years of the menopause that are associated with a net bone loss.

The maintenance of adequate trabecular and cortical bone requires that bone formation and resorption are balanced at the BMU during bone remodeling, such that a high or low level of resorption is usually associated with a similar change in the level of bone formation. The theory that resorption is followed by an equal amount of formation, known as "coupling," refers to the process of information transfer within the BMU, by which the level of resorption within each BMU is matched with an equal amount of bone formation. However, the effects of growth and aging during life, including changes in mechanical stress, mean that this theory of equal bone replacement rarely holds true. During growth there is a positive balance, with the amount of bone replaced at individual BMUs exceeding that lost[2] and with ageing gradual attrition of bone occurs due to a negative balance at individual BMUs.[5] In common metabolic states, such as postmenopausal osteoporosis, while coupling exists and both bone formation and resorption are occurring at a higher level than normal, the amount of bone formed is not equal to that resorbed and bone density reduces. The mechanisms involved in remodeling and coupling have been the subject of a number of recent reviews.[6,7]

Until the early 1980s it was understood that circulating hormones and mechanical forces were the primary regulators of bone metabolism. Although both of these are important, the key influences on both modeling and remodeling are now understood to be locally generated. In this chapter we use parathyroid hormone (PTH) and PTH-related protein (PTHrP) as a prime example of how endocrine and related paracrine factors can affect the skeleton.

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2 PARATHYROID HORMONE AND PARATHYROID HORMONE-RELATED PROTEIN

The main role of PTH in bone metabolism has been long recognized to be maintenance of calcium homeostasis; PTH releases calcium into the circulation from bone and prevents calcium loss through the kidney, and indirectly, increases calcium absorption through the gastrointestinal tract.[8] The fact that hypercalcemia in many cancers reproduced the biochemical features of primary hyperparathyroidism but without immunoreactive PTH, provided the stimulus to search for a tumor-derived factor with similar properties.[9–11]

The discovery of PTHrP as the factor causing the humoral hypercalcemia of malignancy (HHM)[12] revealed a protein that shared a common receptor with PTH. Each protein was shown by both structural and functional studies to have its receptor binding region and activation domain contained within the first 34 amino-acid residues.[12,13] The two proteins were derived by an early gene duplication event from a common precursor. While PTH is an 84 amino-acid molecule, human PTHrP exists as 139, 141, and 173 amino acid isoforms that each arise by alternative mRNA splicing. Of the first 13 residues of PTH and PTHrP, 8 are identical, with no other identities greater than would be expected by chance. The marked conservation of the PTHrP amino acid sequence up to position 111 throughout many species indicates that important functions might reside within this region.

Both PTH and PTHrP initiate their effects by binding with equal affinity to the same G protein-coupled PTH-receptor-1 (PTHR1).[14] Within bone PTHR1 is expressed in cells of the osteoblast lineage, with greater expression in more mature cells, including osteocytes.[15,16] PTHR1 is also expressed in the kidney tubule. Binding of either peptide to PTHR1 results in activation of adenylyl cyclase/cAMP and protein kinase A (PKA) through $G_s\alpha$, and of calcium/inositol phosphate and protein kinase C through the Gq family of signaling proteins.[14] Activation of either signaling pathway confers a unique array of gene expression, with most changes in gene regulation associated with PKA stimulation. To the present time no unique genetic targets of PTHrP versus PTH have been identified in bone.[17]

Continuous exposure to PTH leads to increased bone resorption and bone loss whereas intermittent administration leads to increased bone mass.[18,19] This is the case also for PTHrP treatment, at least as determined by studies using amino-terminal peptides, such as PTHrP(1–36) and (1–34).[20,21] The cellular and molecular mechanisms by which PTH and PTHrP exert their actions will be considered further, and with our focus on endocrine and paracrine influences on bone, we will attempt to put PTHrP into perspective as the local ligand in bone for the PTH/PTHrP receptor (PTHR1).

3 PTHrP IN BONE; PRODUCTION IN OSTEOBLASTS

Evidence for skeletal production of PTHrP came from several sources. PTHrP protein was identified immunologically in normal human rat fetal bone and cartilage[22–24] and PTHrP mRNA was detected in human and rat osteoblastic sarcoma cell lines,[25] in rat osteoblast-rich cultures and preosteoblastic cell lines,[26,27] and in primary cultures of human bone-derived cells.[28] In situ hybridization analyses localized PTHrP to active osteoblasts on the bone surface of newborn rat calvarial sections[26] and also to spindle-shaped cells of the periosteum, which may represent immature preosteoblasts.[26,29] In areas of endochondral bone formation, PTHrP mRNA was detected in the perichondrium and in maturing chondrocytes in a cell-type and stage-specific manner during fetal rat development.[29] In a rabbit model of intramembranous bone formation, PTHrP mRNA and protein were detected at high levels in osteoblastic cells throughout the bone formation process, including in mature, actively synthetic osteoblasts and matrix-embedded osteocytes.[30]

4 PTHrP FUNCTION IN BONE; LESSONS FROM PTHrP NULL MICE

PTHrP null mice were born with severe skeletal abnormalities and died soon after birth, most likely from respiratory failure, due to defective rib cage formation. Their multiple defects in skeletal development indicated the importance of PTHrP in fetal bone development,[31] but made it difficult to assess the role of PTHrP in adult bone. Whereas haploinsufficient PTHrP(+/−) mice were phenotypically normal at birth, by 3 months of age low bone mass was noted, with markedly lower trabecular thickness and connectivity than controls, and an abnormally high number of adipocytes in the bone marrow.[32] The low bone mass was caused by decreased recruitment of bone marrow precursors and increased osteoblast apoptosis compared to wild type. The role of local osteoblast-derived PTHrP in the process of bone formation was confirmed when this phenotype was recapitulated in transgenic mice with an osteoblast-specific knockout of PTHrP.[33] These mice also demonstrated reduced osteoclast formation, likely due to impaired ability of PTHrP-null osteoblasts to support osteoclast formation. Importantly, mice with PTHrP-deficient osteoblasts were normocalcemic, emphasizing that, unlike the systemic action of PTH, PTHrP paracrine action in bone is not required for calcium homeostasis.

These genetic experiments thus revealed actions of PTHrP that reproduce the known effects of therapeutic application of PTH: to stimulate bone formation by promoting the differentiation of committed osteoblast

precursors and by inhibiting apoptosis of mature osteoblasts and osteocytes.[34] The efficacy of PTH as a skeletal anabolic therapy (i.e., one that increases bone mass) has been established for osteoporosis,[35] and depends on intermittent injections, each achieving a sharp peak of PTH in the blood.[36] More sustained elevation of PTH levels favors osteoclast formation through a prolonged elevation in receptor activator of NF-κB ligand (RANKL) production, which promotes osteoclast production from hemopoietic precursors.

What are the ways in which PTHrP can act as a paracrine/autocrine factor in bone? If local PTHrP levels were persistently high, this could favor osteoclast formation by stimulating RANKL production by the osteoblast lineage. Therefore it might be supposed that for PTHrP to enhance osteoblast differentiation and reduce osteoblast apoptosis, as suggested by the phenotypes of the haploinsufficient and osteoblast-specific PTHrP null mice, control mechanisms must exist to ensure that only short-lived, high levels of PTHrP are available to local targets in order to favor bone formation.

We can only speculate about the nature of the PTHrP molecule that gains access to its adjacent target cells. Is full-length PTHrP secreted, and is that the predominant form that interacts locally with target cells? Does its susceptibility to proteolytic breakdown[37] yield shorter products, even in that local environment? Also to be kept in mind is the strong likelihood that biological activities are exerted by those domains of PTHrP released through proteolytic cleavage. This was exemplified in two studies in mice. In one of these, a knock-in of PTHrP(1–84) lacking both the nuclear localization sequence and C-terminal region while retaining the PTHR1-interacting amino-terminal, resulted in multiple abnormalities and early lethality in mice.[38] Homozygous mice exhibited skeletal growth retardation and osteopenia associated with reduced proliferation and increased apoptosis of osteoblasts. These mice also exhibited early senescence with altered expression patterns and subcellular distribution of proliferative- and senescence-related genes in multiple tissues. In the second model, a knock-in of PTHrP(1–66) which excluded a significant part of the midregion, resulted in a similar, but even more severe phenotype.[39] These mouse genetic studies suggest that many of the actions of PTHrP are not mediated by the PTH1R-interacting amino-terminal region; among the generalized abnormalities, absence of the midregion, nuclear localization sequence, and C-terminal region result in greatly impaired commitment and survival of osteogenic and hematopoietic precursors. Mitogenic and antiapoptotic actions of PTHrP nuclear and C-terminal regions have been noted in smooth muscle,[40] chondrocytes,[41] and breast cancer cells[42]; similar actions may occur in osteoblasts with their absence being responsible for the phenotypes observed.

Perhaps PTHrP release would need to be exquisitely regulated in terms of concentration, location, and time so that in bone remodeling it is presented only briefly to its nearby target cells. On the other hand, the spatio-temporal controls might be such that excessive osteoclast formation is much less likely under physiological conditions, where active BMUs are only in place where required for maintenance of the skeleton, and are therefore relatively limited in number. On the other hand when PTH is presented systemically as a pharmacological agent, new and existing BMUs are activated throughout the skeleton. At the end of this chapter we will present an argument for the proposal that paracrine PTHrP in its remodeling role operates at any one time only at those BMUs that are active, whereas systemic administration of PTH (or of analogs) results in widespread BMU activation, with the consequent possibility of resorption activation, and even of hypercalcemia.

5 ANABOLIC ACTIONS OF PTH AND PTHrP

The anabolic effects of intermittent PTH administration on bone and its therapeutic potential in osteoporosis have been extensively studied. There are several mechanisms proposed for the PTH anabolic effect that require its direct action upon the osteoblast lineage. One is the promotion by PTH of differentiation of committed osteoblast precursors,[18] another being inhibition of osteoblast apoptosis,[43] and a third being action upon the osteocyte to inhibit the production of sclerostin, a powerful inhibitor of bone formation.[44] There are also a number of indirect effects of PTH action, which are discussed in more detail later.

The anabolic effect of PTH is the result predominantly of stimulating remodeling by increasing the number and activity of BMUs, with a net increase eventually in the amount of bone at each BMU.[45–47] There is some effect on modeling also, some of this relating to "overfilling" of BMUs.[47] Consistent with the idea of PTH stimulating remodeling are the observations that the anabolic effect of PTH is greater on trabecular and endocortical bone than on periosteal bone.[35,48] The PTH effect in human subjects is particularly marked on the endocortical surface, which remodels very actively in old age.[49] Making a distinction between remodeling and modeling effects of PTH anabolic treatment becomes important when considering the use of PTH in subjects treated with maximally effective resorption inhibitors, where the osteoclast formation and activity essential for remodeling is prevented.[50,51]

Although PTHrP(1–34) has an anabolic effect when administered therapeutically,[20] the pathogenesis of hypercalcemia in HHM largely reflects the bone-resorbing

properties of PTHrP. With the recognition that PTHrP is the paracrine ligand for the PTHR1 in bone,[33,52] several truncated forms including the PTHR1-interacting portion of the molecule have been investigated as anabolic agents, including PTHrP(1–34), PTHrP(1–36), and PTHrP(1–74). The anabolic action of PTHrP(1–36) in human subjects, as assessed by measurement of bone formation markers, has been suggested to be relatively free of the resorptive effect that is an essential component of the remodeling effect of PTH treatment.[21,36,53,54] In these studies of PTHrP(1–36) daily injection, the doses required to increase levels of anabolic markers are many-fold higher than those of PTH(1–34), even though the two appeared to be equipotent in acute infusion studies of their effects on serum calcium and phosphorus, urinary phosphate handling and nephrogenous cAMP.[55] The possibility of PTHrP(1–36) being purely anabolic[54] is an intriguing one, if difficult to explain. It might be due to a difference in pharmacokinetics of the two, with PTHrP(1–36) degraded more rapidly following injection, and thus not so widely distributed to activate BMUs. The result would be that lesser amounts of active agonist would be made available to PTHR1.

Another explanation has been proposed, based on studies of interaction with the PTHR1 of PTH(1–34) and PTHrP(1–36). In cells engineered to overexpress PTH1R, PTHrP(1–36) and PTH(1–34) differed in their initial receptor interaction mechanisms; the action of PTHrP(1–36) was restricted to the cell surface, while PTH(1–34) was more readily internalized and brought about a more prolonged increase in cyclic AMP in the target cells.[56] The authors suggested that such a differential response might explain why daily injection of PTH(1–34) exhibits a resorption response, while PTHrP(1–36) does so to a much lesser extent. The same argument has been applied to a new analog, abaloparatide, which is identical to PTHrP in its first 21 residues, but has 8 residues different from PTHrP between 22 and 34,[57] and which has also been suggested to have a lesser effect on resorption markers than PTH(1–4) in patients.[58] Abaloparatide behaves in a similar manner to PTHrP(1–36) in the study of initial interaction with receptor.[59] These are interesting cell biology findings, but extrapolating them to provide an explanation for in vivo effects is questionable, without any evidence of differences in cellular effects beyond cyclic AMP generation. It is relevant to note that the nature of PTHrP presented to its target physiologically in bone is not known. In the absence of any data there is no reason to suppose that it presents as PTHrP(1–36). The schema proposed in Fig. 17.1 and discussed later in this chapter is relevant to this point.

6 ENDOCRINE PTH, PARACRINE PTHrP; RELATIONSHIPS IN DEVELOPMENT AND POSTNATAL LIFE

Using a variety of validated assays, circulating PTHrP cannot be convincingly detected in normal human subjects (e.g.[60]). That observation gave rise to the view, soon proved to be correct, that any noncancer role for PTHrP must be as a local paracrine factor. It was clear that in the HHM syndrome, PTHrP is secreted by tumors, circulates as a hormone and acts upon bone and kidney.[60,61] In that situation and in lactation where PTHrP is made in the breast and reaches the circulation,[62] the increased circulating PTHrP exerts effects that very closely mimic those of excess PTH. In fetal life, where PTHrP is made by the fetal parathyroids and placenta, it regulates maternal-to-fetal placental calcium transport.[63] In contrast to these

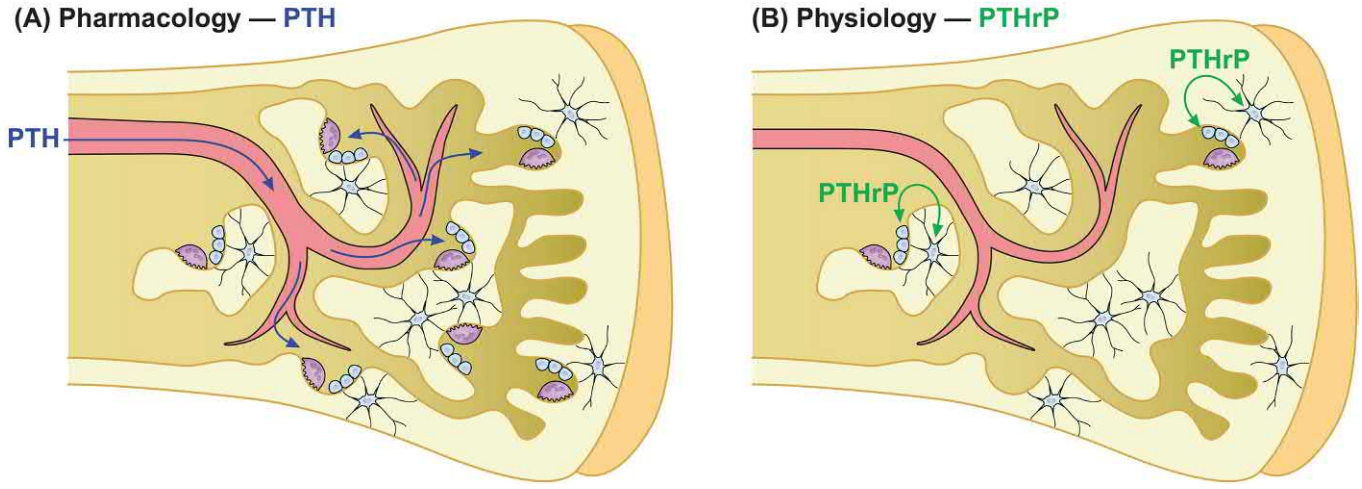

FIGURE 17.1 Contrasting ways in which locally derived parathyroid hormone related protein (PTHrP) and systemically delivered parathyroid hormone (PTH) interact with basic multicellular units (BMUs) in bone remodeling. Whereas systemic PTH is available for widespread activation, resulting in progressively increasing active BMUs, and including dilatation of bone blood vessels (A), locally produced PTHrP acts in those BMUs that are recruited in the normal remodeling process (B). See text for details.

special circumstances in which it functions as a hormone, PTHrP came to be viewed as normally having an autocrine or paracrine role in several different organs of the body.[64,65]

Since the parathyroid glands only develop after mesenchymal condensations are formed at sites of skeletal development, it is the paracrine/autocrine factor PTHrP, secreted locally by chondrocytic cells, that plays the prime role in endochondral bone formation. Studies by Lanske et al.[66] and Vortkamp et al.[67] show that PTHrP and the PTHR1 are downstream effectors of the Indian hedgehog pathway, regulating the correct spatial and temporal progression of chondrocyte differentiation that determines the rate and extent of long bone formation. In this instance, PTHrP has a paracrine role, making use of the PTHR1. A similar paracrine mechanism has been proposed for the skin, where it was shown that PTHR1 is expressed in fibroblasts but not in keratinocytes.[68]

Functional genetic studies have helped to define the relative roles of PTH and PTHrP in calcium homeostasis and bone. The clinical features of hypoparathyroidism in human subjects were recapitulated in adult mice with global ablation of PTH including hypocalcemia and hyperphosphatemia, with increased trabecular and cortical bone volume and a low level of bone remodeling.[69] In contrast, neonatal skeletons had defective growth plate and primary spongiosa development, resulting in less trabecular bone than controls due to decreased osteoblast numbers, as well as hyperporous, nonremodeled cortical bone.[69]

The relationship between the two was investigated further by generating PTH null mice that were haploinsufficient for PTHrP.[70] These mice were still hypoparathyroid, but the PTHrP haploinsufficiency prevented the increase in cortical thickness and reversed the high trabecular bone mass of PTH deficient mice. In this instance, trabecular bone mass was lower than wild type, associated with decreased osteoprogenitor recruitment, increased osteoblast apoptosis, and decreased bone formation. The conclusion therefore was that the PTH null phenotype of increased trabecular bone was caused by increased local production of PTHrP. Whereas in the fetus PTH is essential for bone mineralization and for recruiting osteoprogenitors with locally generated PTHrP being necessary for their future differentiation,[69,70] postnatally, PTH acts on bone primarily to cause resorption and thereby contribute to the maintenance of extracellular fluid calcium. It was clear also in these experiments that PTHrP cannot compensate for the hypocalcemia resulting from PTH insufficiency. Therefore PTHrP does not contribute directly to the maintenance of serum calcium postnatally, but is the main factor stimulating new bone formation, in addition to its actions in development to direct growth plate development by controlling chondrocyte proliferation and differentiation.

Importantly, the mouse genetic studies did not identify an anabolic role for the circulating hormone, PTH, in the adult. Indeed it is difficult to envisage how a circulating hormone could regulate BMUs that are activated asynchronously throughout the skeleton in response to local need. It remains possible though, that circulating PTH could have an anabolic role by influencing bone modeling. Importantly, this could relate to the therapeutic question of whether PTH is capable of any anabolic action when remodeling is totally blocked by powerful resorption inhibitors.[6,50,51]

7 ARE OSTEOCLASTS INVOLVED IN THE ANABOLIC ACTION OF PTH?

The thought that osteoclasts are required for the anabolic action of PTH first arose when the anabolic effect of PTH was significantly reduced in sheep coadministered with a bisphosphonate (tiludronate) as an inhibitor of bone resorption.[71] Treatment of osteoporotic patients concomitantly with PTH and a bisphosphonate resulted in significant early blunting of the anabolic response to PTH,[72,73] with full PTH responsiveness eventually returning after treatment was stopped. Some, but not all studies of the PTH anabolic effect in rats treated concomitantly with bisphosphonates have also shown impaired anabolic responses.

If an osteoclast function is required for the anabolic effect of PTH, how is that connection made since osteoclasts do not express a functional PTH receptor? In rats a single subcutaneous injection of PTH resulted in a transient increase in mRNA for RANKL and a decrease in that for OPG, with maximum effect at 1 h that returned to control within 3 h, contrasting with a persistent elevation of RANKL mRNA observed with a 6 h infusion of PTH.[74] This led to the suggestion that a subtle or transient increase in osteoclast formation or activation might be needed to prepare the bone surface for new matrix deposition. The demonstration that PTH could rapidly stimulate osteoclasts came 30 years ago,[75] showing that intravenous injection of PTH in young rats resulted in transient activation of osteoclasts in vivo, evident within 30 min, and followed only some hours later at high PTH doses by increased osteoclast number. The rapid nature of this response might be explained by the existence of quiescent osteoclast precursors in the proposed osteoclast niche.[76]

Observations in genetically manipulated mice and from human genetics suggest that the osteoclast itself could also be the source of an activity that contributes to the fine control of osteoblast function in bone remodeling,[77,78] and that this might contribute to the anabolic action of PTH. In mice with a high level of bone remodeling induced by a mutation in the receptor used by all

IL-6 family cytokines, glycoprotein 130 (gp130$^{Y757F/Y757F}$ mice) low trabecular bone mass was observed because the level of resorption was relatively greater than that of formation. Thus the coupling process was dissociated in a manner resembling that of estrogen withdrawal. gp130$^{Y757F/Y757F}$ mice crossed with IL-6 null mice had similarly high osteoclast numbers and increased bone resorption, however these mice showed no corresponding elevation in bone formation and thus had extremely low bone mass. This indicated that stimulation of bone formation coupled to the high level of bone resorption in gp130$^{Y757F/Y757F}$ mice was an IL-6-dependent process, but not necessarily showing that it is mediated by IL-6 itself.[79]

Furthermore, in mice deficient in either c-*src*,[80] Pyk2,[81] cathepsin K,[82] or the chloride-7 channel (ClC-7),[83] bone resorption is inhibited without inhibition of bone formation. In each of these knockout mouse lines osteoclast resorption is greatly reduced by the mutation, although osteoclast numbers are not. Indeed in all these cases osteoclast numbers are actually increased, possibly because of reduced osteoclast apoptosis.[84,85] A possible explanation of the uncoupling of resorptive activity and bone formation in these mice is that these osteoclasts, although unable to resorb bone, remain capable of generating a factor (or factors) required for bone formation. This is illustrated by the findings that *c-src*$^{-/-}$ mice retain an anabolic response to PTH injections,[86] whereas mice lacking c-*fos*, which are unable to generate osteoclasts, have reduced bone formation as well as resorption, and show a marked blunting of the anabolic action of PTH.[87]

The foregoing led to the suggestion that what is needed for full expression of the anabolic response to PTH, in addition to its direct effect on the osteoblast lineage, is a transient effect on the osteoclast, achieved by promoting activation, but not necessarily formation of new osteoclasts (Fig. 17.2).[17,77] A number of candidate osteoclast products, termed "coupling factors" have been proposed for this role.[7,88–92] One such pathway is EphrinB2–EphB4, which was suggested following the finding that osteoclast-derived ephrinB2 acts through a contact-dependent mechanism on EphB4, its receptor in the osteoblast, to promote osteoblast differentiation and bone formation.[93] If such a coupling mechanism were to operate it would require many sites of contact between active osteoclasts and differentiating osteoblasts; this appears to occur very uncommonly. Further, the dramatic upregulation of osteoblastic expression of EphrinB2 by PTH suggested that the interaction of EphrinB2 and EphB4 may also be important for the anabolic action of PTH.[17] However, it seems that the need for EphrinB2–EphB4 signaling in the context of PTH action is not one where communication between osteoblasts and osteoclasts is required. In contrast, it is the interaction between EphrinB2 and EphB4 within the osteoblast lineage that is most important. This was established by showing that the PTH anabolic effect was largely blunted both in mice with osteoblast-specific ablation of EphrinB2,[94] and in mice treated with a systemic

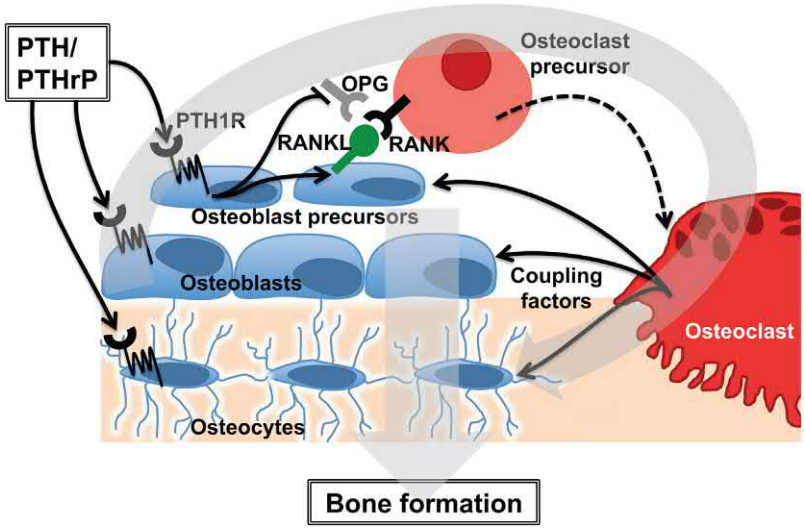

FIGURE 17.2 The communication cycle between osteoblasts and osteoclasts that contributes to PTH and PTHrP anabolic action. PTH and PTHrP act on the PTH receptor (PTH1R) expressed in the osteoblast lineage in committed osteoblast precursors, bone forming osteoblasts and matrix-embedded osteocytes to stimulate bone formation. In addition, PTH receptor action stimulates production of receptor activator of NF-κB ligand (RANKL) and inhibits production of the RANKL decoy receptor osteoprotegerin (OPG). RANKL interacts with its receptor (RANK) on osteoclast precursors to stimulate their differentiation into bone-resorbing osteoclasts. Osteoclasts provide a range of factors, both proteins produced by osteoclasts and growth factors released by their resorption of bone matrix. These coupling factors stimulate bone formation by actions on each stage of osteoblast differentiation. The sum of both the direct action of PTH on the osteoblast lineage, and the stimulation of osteoclast-derived coupling factors, is an increase in bone formation.

inhibitor of the EphrinB2–EphB4 interaction.[95] Further to this, blocking the interaction between EphrinB2 and EphB4 in vitro within the osteoblast lineage reduces osteoblast differentiation and the mineralizing capacity of osteoblastic cells.[17,96] In vivo evidence consolidating this view of the importance of EphrinB2–EphB4 interaction within the osteoblast lineage, not only in the context of PTH anabolic action, but also during normal bone physiology, was that knockout of EphrinB2 in the osteoblast lineage using an Osx-Cre promoter resulted in mice with delayed osteoblast differentiation, increased apoptosis, and reduced mineralization.[94]

Other possibilities for osteoclast-derived factors that could promote osteoblast differentiation include sphingosine-1-phosphate, BMP-6, and Wnt 10b.[88] It might be noted that Wnt10b production by T cells in response to PTH is offered as the explanation for the T cell-mediated effect of PTH on bone formation (see later).[97] Another possibility is cardiotrophin-1 (CT-1), a member of the family of cytokines that signal through the gp130 transducer. This cytokine is expressed in differentiated osteoclasts but not in the osteoblast lineage.[98] As well as indirectly stimulating osteoclast differentiation through stimulation of RANKL production by stromal cells, CT-1 powerfully stimulates bone formation. CT-1, like oncostatin M (OSM), leukemia inhibitory factor (LIF), and PTH, also profoundly decreases sclerostin mRNA expression by osteocytes,[99] thus introducing the concept that osteoclast products might communicate with the osteoblast lineage by signaling directly to the osteocyte (Fig. 17.2).

Whether the osteoclast, either by its resorptive actions or by its production of factors that influence osteoblasts, contributes significantly to the anabolic action of PTH remains a matter for further investigation.

8 GROWTH FACTORS IN THE LOCAL ACTIONS OF PTH AND PTHrP

The role of locally generated growth factors in bone has been explored extensively since the suggestion that bone formation might be coupled to bone resorption by the release of bone forming factors from the bone matrix during resorption (Fig. 17.2).[100] Among matrix substances that could promote osteoblast mitosis or stimulate bone formation in vivo were insulin-like growth factor (IGF) I and II, acidic and basic fibroblast growth factor (FGF), transforming growth factor β (TGFβ) 1 and 2 and TGFβ heterodimers, bone morphogenetic proteins (BMPs) 2, 3, 4, 6, and 7, platelet-derived growth factor, and probably others.[101–104] Most attention has been paid to TGFβ and IGF-1.

Although liver IGF-1 is regulated largely by growth hormone secreted from the pituitary, this is not the case for IGF-1 production in nonliver tissue, such as bone. IGF-I and IGF-II are produced by osteoblasts, and this production is enhanced by PTH stimulation of bone formation.[105,106] When injected together with the IGF-binding protein IGFBP-3 into rats, IGF-I was reported to increase bone volume.[107] Ablation of the IGF-1 receptor in mice resulted in decreased bone mass, despite increased osteoid volume and surface, establishing a likely important role for IGF-1 in bone mineralization.[108] In these mice, and in mice with either global or osteoblast-specific IGF-I deficiency, both the anabolic and catabolic responses to PTH were significantly impaired.[109,110] The conclusion from the mouse studies is that IGF-1, generated locally by cells of the osteoblast lineage, has a significant part to play in the anabolic effect of PTH. The anabolic IGF1 action has been specified to show stimulation of differentiation of recruited stromal stem cells by activating mammalian target of rapamycin (mTOR).[111] The concept surrounding involvement of IGF1 in PTH action has been extended by recent evidence that PTH acts upon osteoblast lineage cells to stimulate aerobic glycolysis, that is, increased glucose consumption and lactate production in the presence of oxygen.[112] This effect is secondary to the increased IGF1 in response to PTH, and notably, pharmacological blockade of glycolysis suppressed the bone anabolic effect of intermittent PTH. The aerobic glycolysis effect and the further link to IGF1 raise interesting questions concerning the local physiological mechanism of PTHrP action, in particular, whether a PTHrP/IGF1 relationship operates in activated BMUs, switching osteoblast lineage cells to produce energy through aerobic glycolysis.

The link between TGFβ and the anabolic effect of PTH is a strong one. When TGFβ is injected next to the periosteum or endosteum, there is a substantial increase in local bone formation in rats and other species.[113,114] Although the local balance is clearly positive, there is also an increase in endocortical bone resorption at the same time. Thus, like IGF-I, and TGFβ seems to stimulate both resorption and formation. Indeed, transgenic overexpression of TGFβ in mice resulted in high turnover bone loss.[115]

Bone is an abundant source of TGFβ. It was proposed that TGFβ, which is produced as an inactive precursor in bone by osteoblasts,[116] is stored as an inactive precursor in the matrix and activated by proteolytic cleavage accomplished by the acid resorptive environment.[117] TGFβ is produced by all osteoblastic cells examined and its production is increased by PTH. The PTH anabolic effect in bone was accompanied by progressive increases in IGF-1 and TGFβ,[105] leading the authors to suggest that both growth factors play roles in the anabolic effect of PTH. Some support for this comes from more recent evidence that active TGFβ1 released during bone resorption couples bone formation to resorption by inducing the migration of bone mesenchymal stem cells to sites of prior resorption, thus making them available for

differentiation and bone formation in remodeling.[118] This would provide an attractive explanation for the concept that osteoblasts are recruited for remodeling from a pool of stem cells, and need to be attracted to remodeling sites to be differentiated and replenish the osteoblast population. Consistent with this idea, when bone resorption was inhibited with a bisphosphonate, so too was the release of active TGFβ and the recruitment of Sca-1+ve stem cells to remodeling sites.[119] This was associated with decreased bone formation. It remains difficult to see how tight quantitative control of the amount of active TGFβ can be exercised if it depends solely on the acidic pH at resorption sites. It may be that the necessary quantitative control is left to the next stage—influences upon the stem cells when they reach the remodeling site. Furthermore, while an action of TGFβ to promote stem cell migration to appropriate sites is plausible, such an effect might contribute to the overall process by which bone formation follows bone resorption. It fits less easily as a mechanism for rapid changes in bone remodeling.

A further level of regulation arises from evidence of an even more intimate association between PTHR1 and TGFβ receptor signaling, with the findings that the TGFβ receptor II (TβRII) can phosphorylate the PTHR1, switching it off by promoting its endocytosis as a PTH1R–TβRII complex.[120] Supporting this model, deletion of TβRII in osteoblasts increased cell surface PTHR1 and signaling, resulting in a bone phenotype similar to that of mice with constitutively active PTHR1.

These findings indicate that active TGFβ is generated at resorption sites, possibly enhanced by PTH/PTHrP, that skeletal stem cells can respond to this TGFβ by mobilizing to remodeling sites, and that TGFβ signaling might control the extent of PTH/PTHrP signaling. These functions answer some of the questions that should be considered regarding the role of local growth factors in the hormone- and cytokine-mediated coupling of bone formation to bone resorption: (1) Which cells produce them and under what circumstances? (2) Do they stimulate bone formation in vivo? (3) Can they be released from the matrix in active form and in controlled amounts during bone resorption? (4) Is there any evidence for an increase in the abundance of these active factors at sites of bone remodeling? and (5) are there regulated mechanisms by which they are activated?

9 gp130 CYTOKINES AS AGENTS OF LOCAL CONTROL OF PTH ACTION

gp130 is a receptor subunit capable of intracellular signaling that is required for the cellular action of the IL-6 family of cytokines. The most well-known are interleukin IL-6 and IL-11, LIF, CT-1, OSM, and ciliary neurotrophic factor. In addition to contributing to inflammation, gp130 signaling cytokines also function in the maintenance of bone homeostasis. Expression of each of these cytokines and their ligand-specific receptors is observed in bone and joint cells,[121,122] and bone-active hormones including PTH and inflammatory cytokines regulate their expression by osteoblasts.[123] gp130 signaling cytokines have been shown to promote the differentiation and activity of osteoblasts, osteoclasts, and chondrocytes. Furthermore, gene-knockout mouse models of cytokines and receptors in this family have identified distinct roles for each of these cytokines in regulating bone resorption, bone formation, and bone growth, and there is much evidence for their roles in the pathogenesis of bone and joint disorders.[124]

The initial interest in gp130 signaling in bone came from its role in the promotion of osteoclast formation. Together with cyclic AMP/PKA and the vitamin D nuclear receptor, gp130 signaling comprised the third major pathway in osteoblastic stromal cells capable of generating osteoclasts in cocultures with hematopoietic cells,[125] ultimately shown to be due to promotion of RANKL production.[126] Thus in such cocultures, treatment with IL-6 and its soluble receptor (sIL-6R) induced osteoclast formation, as did IL-11, OSM, CT-1, and very mildly, LIF, all of which use gp130 as a common transducer.[127,128] When cells from IL-6R-overexpressing transgenic mice were used in crossover cocultures with hematopoietic cells from wild-type mice, the expression of IL-6R by osteoblastic cells was shown to be indispensable for the induction of osteoclasts by IL-6.[129] This clearly demonstrated that, like PTH, stimulation of osteoclast formation by IL-6 required direct action on the osteoblast. A central role of the gp130-coupled cytokines in osteoclast development stimulated by a range of hormones and cytokines was further indicated by the observations that PTH; $1,25(OH)_2D_3$; PGE_2; IL-1; and TNFα all promoted IL-11 production by osteoblastic cells (Fig. 17.3)[130] and addition of neutralizing anti-gp130 to cocultures fully blocked stimulation of osteoclast formation by IL-1, and partly blocked osteoclast formation in response to PTH; $1,25(OH)_2D_3$; and PGE_2.

In mice null for individual ligands or receptor components involved in gp130 signaling, the osteoclast phenotype varies. Adult IL-6 null mice demonstrate normal numbers of osteoclasts, indicating that this cytokine's role in osteoclastogenesis can be compensated for by other factors. However, IL-6 appears to be required for the increased osteoclastogenesis observed in ovariectomy (see later),[131] in catabolic PTH infusion, where IL-6 potentiated the bone-resorbing effect of PTH[132] and in bone destruction in inflammatory arthritis.[133] Genetic deletion of CT-1, LIF, and LIFR all lead to increased osteoclast formation in vivo,[98,134,135] while osteoclast numbers are low in IL-11R and oncostatin M receptor (OSMR) deficient mice.[121,136] This indicates that these cytokines are

required for normal osteoclastogenesis and normal bone remodeling, and their roles are not redundant.

While osteoclasts express only gp130 and IL-6R and respond to IL-6 with increased STAT3 phosphorylation,[137] both osteoblasts and osteocytes respond to many members of this family since they express not only gp130, but also many of the ligand-specific subunits required for cytokine action (IL-6R, IL-11R, CNTFR, LIFR, and OSMR).[99,138,139] OSM, CT-1, IL-11, and LIF all stimulate osteoblast differentiation from stromal cells, and at the same time reduce the ability of these cells to differentiate into adipocytes (Fig. 17.3).[98,121,140] Even though OSM, LIF, CT-1, and IL-11 have the same action on cells in culture, their effects on osteoblast and adipocyte differentiation in vivo are not redundant, since CT-1, LIF, IL-11R, and OSMR deficient mice all demonstrate a low level of bone formation in vivo and increased adipocyte volume within the bone marrow space.[98,121,136,140] The nonredundant roles of these cytokines may stem from the cell-types and conditions under which these cytokines are expressed. For example, in the BMU, CT-1 is expressed only by the osteoclast, while OSM is not expressed in osteoclasts, but is expressed in all osteoblast-lineage cells including osteocytes, as well as activated macrophages within the bone marrow.[141]

OSM, CT-1, and LIF, like PTH, have also been reported to strongly inhibit osteocytic expression of sclerostin,[121] an essential and powerful inhibitor of bone formation that acts as a Wnt signaling antagonist.[142] Although OSM is capable of signaling through a receptor complex containing either LIFR or OSMR, it appears that its influence on both sclerostin expression and bone formation is mediated specifically by LIFR, while its influences on osteoblast/adipocyte commitment and osteoclastogenesis are mediated by OSMR.[121] This receptor-specific divergence of influence of a single cytokine on osteoblasts and osteoclasts appears to be unique to OSM; LIF and CT-1 both stimulate osteoclast formation and inhibit sclerostin through the LIFR.[121] The specific structural interaction between OSM and the LIFR compared to the interaction of LIFR with its "native" ligands CT-1 and LIF is not yet solved.

OSM uses the OSMR to powerfully induce RANKL production and osteoclast formation.[121] For that reason, OSMR–null mice were used to study the anabolic action of PTH. In these mice, the duration of elevated RANKL expression after PTH treatment is prolonged, although the mechanism by which this occurs remains unknown. The result is that intermittent treatment with PTH in vivo resulted in increased bone resorption that negated the anabolic effect of PTH and resulted in bone loss.[123] This indicates that delivery of PTH in an anabolic mode can be readily converted to a catabolic outcome if the stimulation of RANKL and osteoclastogenesis is sufficiently prolonged.

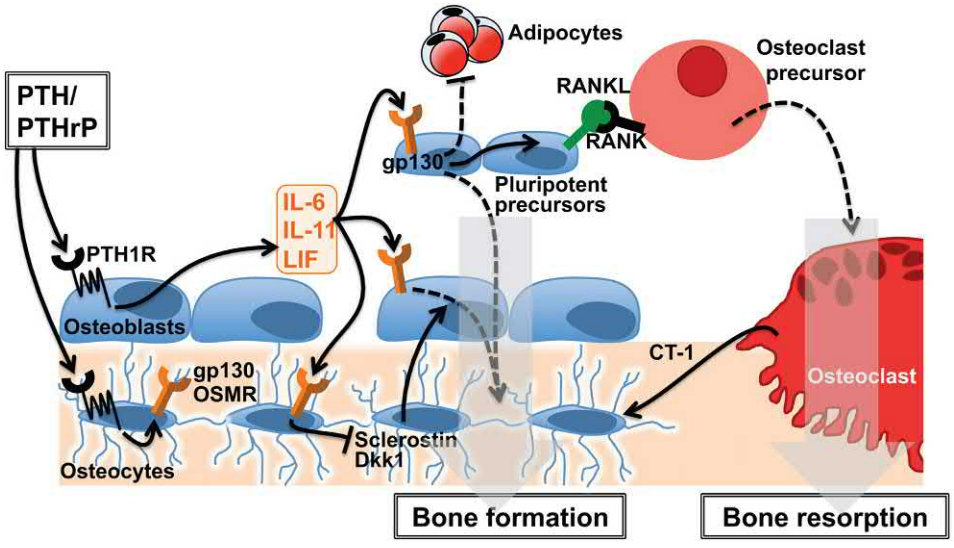

FIGURE 17.3 Contribution of the interleukin 6 (IL-6) family to PTH and PTHrP action in bone. PTH and PTHrP act on the PTH receptor expressed in the osteoblast lineage in committed osteoblast precursors, bone forming osteoblasts, and matrix-embedded osteocytes. This action stimulates production of IL-6 family cytokines including IL-6, interleukin 11 (IL-11) and leukemia inhibitory factor (LIF), as well as promoting expression of the oncostatin M (OSM) receptor (OSMR) and the common receptor for the entire IL-6 family (gp130) in the osteoblast lineage. These locally produced cytokines also act at all stages of osteoblast differentiation. In pluripotent osteoblast precursors they inhibit adipocyte differentiation and promote osteoblast differentiation and bone formation. IL-6 family cytokines promote production of receptor activator of NF-κB ligand (RANKL) that interacts with its receptor (RANK) on osteoclast precursors to stimulate their differentiation into bone-resorbing osteoclasts. IL-6 family cytokines, including cardiotrophin-1 (CT-1) derived from osteoclasts, also act on osteocytes to inhibit production of sclerostin and Dkk1 (Wnt pathway inhibitors); this also promotes bone formation. The action of IL-6 family cytokines on osteocytes is required for PTH anabolic action.

Since IL-6 family cytokines act on osteocytes to inhibit sclerostin expression and signal directly through osteoblasts to promote both bone formation and osteoclastogenesis, it was of interest to determine whether osteoblast-lineage expression of gp130 was required for normal osteoclastogenesis and response to PTH. However, when gp130 was genetically ablated from the osteoblast lineage or in osteocytes (using Osx1Cre or Dmp1Cre, respectively) no significant alteration in osteoclast numbers was observed.[143] In contrast, both mouse models exhibited low trabecular bone mass and reduced cortical material strength due to impaired osteoblastogenesis.[143] In addition, deletion of gp130 in committed osteoclasts again did not result in reduced osteoclast formation, but bone formation was reduced, indicating that any direct effect of IL-6 on osteoclasts promoted its ability to signal to osteoblasts, rather than promoting the activity or differentiation of osteoclasts themselves.[137] Finally, when mice deficient in gp130 in osteocytes were treated with PTH, no anabolic action was observed, either in trabecular or cortical bone.[144] This reduction in PTH anabolic action was associated with reduced expression of PTH1R in osteoblasts lacking gp130 both in vivo and in vitro, which suggests that IL-6 family cytokines promote PTH1R expression. Consistent with this, reductions in the Wnt inhibitors sclerostin and Dkk1, normally associated with PTH treatment were not observed in the gp130-deficient mice (Fig. 17.3). Surprisingly however, the rapid elevations in mRNA levels of RANKL and IL-6 were retained, suggesting that the effect of PTH on osteoclastogenesis may be mediated by osteoblasts at an earlier stage of differentiation than the cells that mediate its anabolic action.

10 SCLEROSTIN AS A LOCAL FACTOR PROMOTING PTH ACTION

As is the case with the gp130 cytokines, downregulation of sclerostin by PTH provides another mechanism through which PTH has its anabolic action. Such a role for sclerostin has been indicated by an impairment in the PTH anabolic effect in both sclerostin deficient, and overexpressing mice,[145] although more recent work suggests this role may be specific to cortical bone.[146] Similar observations have been made in mice that lack Dkk1, another Wnt signalling inhibitor suppressed by PTH.[147] Since one of the major actions of Dkk1 and sclerostin is to prevent the low-density lipoprotein receptor-related protein 5 (LRP5) from contributing to Wnt signaling in bone,[148] it was surprising that the PTH anabolic effect was also not observed in LRP5 deficient mice.[149,150] Furthermore, in one of these studies the osteogenic response to mechanical loading was very greatly reduced in mice LRP5$^{-/-}$ mice.[150] Thus, despite the inadequate processing of a mechanical response to loading, PTH response was maintained, suggesting that the contribution of reduced Dkk1 and sclerostin to the anabolic action of PTH may be independent of LRP5.

Given the dramatic effect of PTH on sclerostin expression, and its contribution to the anabolic action of PTH, it was initially surprising to note a report describing osteocyte-specific deletion of the PTHR1 without a dramatic bone phenotype; only a barely detectable reduction in bone mass was reported.[151] These mice did demonstrate significant hypocalcemia, both under normal conditions, and when hyperparathyroidism was induced by a low calcium diet. This provoked the intriguing suggestion that PTHR1 in osteocytes may regulate calcium homeostasis, either by postulated release of calcium by osteocytic osteolysis, or by osteocyte-mediated influences on the gut or kidney. This may involve fibroblast growth factor 23 (FGF23), a phosphaturic hormone that is produced by osteocytes. Circulating FGF23 levels are increased in hyperparathyroidism.[152] Furthermore, genetically altered mice with constitutively active PTHR1 in osteocytes demonstrate elevated circulating FGF23 levels.[153] This possibility that calcium and phosphate homeostatic effects of PTH may be mediated by osteocytes requires further exploration. The regulation of sclerostin production by gp130 cytokines, particularly OSM[121] might provide an adequate alternative pathway if PTH/PTHrP signaling is blocked.

More recent work on a larger sample size than the initial preliminary study[153] is even more surprising; osteocyte-specific deletion of PTHR1 led to a significant elevation in trabecular bone mass.[154] As expected, these mice exhibited blunted responses to both anabolic and catabolic modes of PTH administration, and blunted sclerostin and RANKL gene responses, confirming a role for osteocytes in these actions of PTH. However, the increased trabecular bone mass and cortical thickness in untreated adult animals suggests that the combination of PTH and PTHrP signaling through the PTHR1 limits accrual of trabecular bone. The increase in bone mass was not associated with any significant alteration in osteoblast or osteoclast numbers, but paradoxically, sclerostin expression was elevated and collagen I mRNA levels were significantly reduced. The cause of the high bone mass phenotype in these mice remains unknown.

11 OTHER INFLUENCES OF PTH/PTHrP ON BONE THROUGH THE BONE MARROW MICROENVIRONMENT

The interaction between the cells on the bone surface and cells within the bone marrow microenvironment is a rapidly developing field, where interactions

between these cells are now understood to be required for maintenance of normal bone volume, and osteoblasts are required for maintenance of the hematopoietic stem cell niche.[155] There is evidence for PTH action on hemopoiesis dating from the 1950s when parathyroid extract was found to enhance the survival of irradiated rats.[156] Later studies showed that hemopoietic defects in thyro-parathyroidectomized rats were rescued with administration of PTH,[157,158] as also was the delayed liver regeneration following partial hepatectomy.[159] More recently, mice with osteoblast-specific expression of a constitutively active PTHR1 demonstrated not only an increase in bone formation and osteoblast number,[160] but also a stromal-cell dependent increase in long-term repopulating hemopoietic stem cells.[161] The same effect on hemopoiesis was observed with exogenous PTH treatment.[161] No hematopoietic defect was noted in mice null for PTHrP in osteoblasts,[33] so the question arise whether it is PTHrP or PTH that has a physiological role in maintaining the HSC niche remains unanswered.

There is evidence also that actions of PTH/PTHrP on bone formation may depend on contributions from other bone marrow populations. For example, T cells have been shown to potentiate PTH-induced cortical bone loss through CD40L signaling,[162] and PTH acts through its receptor on T cells to promote the production of Wnt 10b, which in turn increases osteoblast differentiation and bone formation.[97]

12 THE PTH–PTHrP RELATIONSHIP IN VASCULATURE AND BONE

The discovery of PTHrP in 1987 provided many pathways that have led to deeper understanding of the physiological roles of PTH, in particular revealing that endocrine PTH and paracrine PTHrP engage cooperatively to regulate bone and calcium metabolism, with their interactions at their most complex during early development. We are learning how the hormone, PTH, and the structurally related paracrine factor, PTHrP, share the use of their common receptor in many tissues, with the focus in this chapter on bone and cartilage. The favored current concept is that PTH the hormone regulates calcium homeostasis in development and maturity. PTHrP on the other hand acts during skeletal development to control chondrocyte proliferation and differentiation, and, of these two proteins, PTHrP is the main factor generated locally in the postnatal skeleton and acts through the PTHR1 in bone remodeling, but does not normally contribute to the maintenance of serum calcium. These views have developed largely as a result of the insights provided from mouse genetic experiments (discussed earlier), but much is owed also to pharmacological studies in animals, and to the therapeutic application of PTH in osteoporosis. We might now regard PTH in its application as a skeletal anabolic therapy as a pharmacological reproduction of the local physiological action of PTHrP. This sharing of function between a hormone and its related cytokine is reminiscent of the relationship between growth hormone and IGF-1. As in that case, the relationship is functional in many tissues, and a particularly instructive example comes from the actions of PTHrP and PTH on the vasculature smooth muscle beds.

It had been known since the 1920s that injection of PTH (or parathyroid extract in the early years) resulted in dose-dependent increases in blood flow through a range of vascular beds, accompanied by decreases in blood pressure,[163–165] (reviewed in Ref. 166). At the time these effects, that were observed in many animal species, did not fit easily with what was known for the physiological role of PTH to control blood calcium levels.[163] With the discoveries that followed rapidly after the isolation of PTHrP it became clear that this was a reflection of a local physiological role of PTHrP, which is produced in smooth muscle beds of the stomach and intestine, uterus, urinary bladder, and arterial vessels, acting in all those tissues as a smooth muscle relaxant (reviewed in Refs. [64,65]). PTHrP expressed in smooth muscle acts rapidly to relax the vasculature[167] through an endothelium-independent mechanism, and vasoconstrictors, such as angiotensin II induced a rapid rise in PTHrP production.[168] Thus increased PTHrP production following vasoconstriction could provide a mechanism to limit or reverse this effect through the relaxant action of PTHrP on smooth muscle.

If PTHrP does indeed have a physiological role as a local regulator of vascular smooth muscle tone, being generated and acting in response to local needs, it must be very different when widespread engagement of the vascular PTHR1 receptors takes place with systemic PTH administration. The pharmacological response is a generalized one, with widespread dilatation of vascular beds, increased total blood flow, and decreased blood pressure.[164,165] As in bone, the effects of PTH on vasculature vary with the mode of pharmacological administration: while intermittent PTH increased bone vascular perfusion by approximately 30%, and almost doubled microvessel size, continuous infusion of PTH did not modify vascular perfusion, but reduced microvessel size.[169] Whether these opposing effects on microvessel size might determine the level of osteoclast formation in response to intermittent versus sustained high levels of PTH remains unknown.

The difference between PTH and PTHrP action in bone is not dissimilar to that in the vasculature. The work of Miao et al.[33] shows convincingly in the mouse that haploinsufficiency of PTHrP results in bone loss, and that genetic ablation of PTHrP in the osteoblast lineage recapitulates this deficiency, resulting in a low

turnover bone loss, with reductions in both, bone formation and resorption, but without any change in calcium homeostasis. This low bone remodeling result is determined by changes in PTHrP production locally in bone that influence the behavior of BMUs in the bone remodeling process.

Bone remodeling takes place at many discrete sites throughout the skeleton, but does so asynchronously, and in response to the requirements of responses to loading, of repair of damage, and of replacement of old bone. When PTH is used by intermittent (daily) injection to engage the PTHR1 in bone to increase bone formation, widespread activation of BMUs takes place (Fig. 17.1). This is analogous to the situation described earlier for vascular effects, and both of these are far removed from physiology. The pharmacokinetics required for an anabolic response to PTH is that the peak circulating level of hormone should be short-lived.[36] If the decline in PTH levels is sufficiently prolonged, the dominant effect becomes that of increased osteoclast formation, and hence increased bone resorption. There are several examples for the same, including primary hyperparathyroidism, the prolonged PTH release noted in attempts to develop calcilytics as bone-forming agents,[170] and the prolonged PTH activation in OSM receptor-deficient mice.[123] Further, in mice in which the *PTHR1* is mutated to result in lifelong persistent activation of the PTHR1, the result is increased trabecular bone formation and resorption, especially in cortical bone.[160]

If this portrayal of the PTH–PTHrP relationship holds true, it might be expected that repeated generalized recruitment of BMUs by the daily injection regimen would so increase remodeling activity that this would be reflected in increased circulating and excreted resorption markers. This is what happens within some months of starting PTH intermittent injection therapy.[35] A further corollary relates to the nature of the local PTHrP action. In discussing the mouse genetic data of Karaplis and coworkers, it was suggested that there must be local mechanisms that ensure only brief exposure of the PTHR1–PTHrP, and that this might be achieved by a combination of the short half-life of PTHrP mRNA and the great susceptibility of the protein to proteolytic cleavage.[52] However, this might not be relevant. Whatever the nature of locally derived PTHrP that activates the PTHR1 in remodeling—whether full length protein or a shorter proteolytic product—its duration of action at the remodeling site might not be so crucial, since only a limited number of remodeling sites are involved at any one time in the physiological process, and there is consequently little prospect of prolonged stimulation promoting a generalized increase in resorption.

This discussion is relevant to that earlier, concerning the suggested purely anabolic action of PTHrP(1–36).[54] Explanations offered for this are either that PTH and PTHrP(1–36) have different pharmacokinetics,[171] or that PTH action at the receptor is more prolonged than that of PTHrP(1–36), for which there is evidence[56] (vide supra). The reason behind this is that an effect through the PTHR1 at the BMU of as short a duration as possible could avoid stimulating of osteoclast formation and activity. In contrast, the repeated injection of PTH(1–34) or PTH(1–84) would eventually lead to increased resorption markers.[35,172]

This apparent requirement of a brief exposure of the PTHR1 to agonist in order to achieve an anabolic response, has become a standard view. For example, attempts to develop anabolic therapies by using calcilytic agents to release PTH from the parathyroid gland, have aimed at achieving short-lived peaks of circulating PTH. So far these attempts have not been successful.[170,173,174]

13 CONCLUSIONS

In conclusion, PTH and PTHrP appear to have complementary roles in adult physiology. Of the two, PTH is the primary physiological agent that regulates calcium homeostasis through an endocrine mechanism, while paracrine PTHrP acts at the BMU on osteoblast lineage cells to regulate bone formation and bone resorption. A greater number of BMUs are recruited by the pharmacological action of PTH arriving from the bloodstream (Fig. 17.1). The sheer number of BMUs now active in the bone allows PTH to increase the amount of bone, but there is also a progressive resorptive effect. The physiological action of PTHrP on the other hand is confined to those relatively few BMUs needed at any point of time for engagement in remodeling.

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CHAPTER

18

Genetics of Bone Fat and Energy Regulation

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1 INTRODUCTION

In the 21st century, two sharply contrasting disorders of body composition, obesity and osteoporosis, have reached near epidemic proportions and challenged both our scientific and our financial resources. The growing prevalence of these diseases has also enabled investigators to explore new perspectives on the molecular, cellular, and genetic determinants that regulate body composition and energy homeostasis shared by both disorders.[1] This, in turn, has resulted in a sea-change in our understanding of the multiple mechanisms controlling body mass and energy utilization. Shared regulation of bone and fat occurs at several levels, starting with genetic determinants that can affect cellular actions in bone, adipose tissue, and the central nervous system (CNS). Acquisition and maintenance of bone and fat are also mediated through central and peripheral mechanisms including multiple endocrine and paracrine determinants.[2] Importantly, the hypothalamus is the principal controller of these target tissues through neuronal [e.g., sympathetic nervous system (SNS)] and hormonal mediators.[3] In the case of the former, the hypothalamus modulates fat and bone tissue via two major pathways: (1) sympathetic nervous system activation that modulates appetite, insulin sensitivity, energy utilization, and skeletal remodeling; (2) paracrine and endocrine hormonal factors secreted by the hypothalamus. In respect to the latter, paracrine pituitary-activating hormones, such as CRH and TRH can modify metabolic homeostasis through their secondary target glands, the adrenals and thyroid. Similarly the hypothalamus can secrete factors, such as FGF-21, BDNF, oxytocin, Neuromedin U, NPY, melatonin, and IL-6 that impact bone and fat in a systemic fashion.[4,5] Circulating serum factors, such as TNFα and PTHrP, may also play a role in determining how the body utilizes fuel and distributes it to other organs, particularly in states of cachexia.[6] Muscle derived growth factors can have an impact on bone and adipose tissues, particularly inguinal depots in rodents (or subcutaneous depots in humans). Currently one of the most exciting myokines is irisin; a cleaved protein product from muscle after exercise that induces beiging of white adipose tissue (WAT) and also positively impacts cortical bone.[7]

In addition to shared-regulatory determinants between bone and fat, as well as a common central signal from the hypothalamus, bone, and fat cells arise from the same mesenchymal derived progenitor cell, primarily in the bone marrow[8] (Fig. 18.1). There is emerging evidence of significant plasticity between these two lineages, and some have suggested there may be transdifferentiation of these terminally differentiated adult cells. Cell fate decisions in the bone marrow are critical for defining total fat and bone mass particularly since recent studies have hinted that around 10% of adipocytes in peripheral depots may originate from the marrow after bone marrow transplantation.[9] Cell fate within the marrow is also particularly relevant in cases where fuel supplies are low; for example, anorexia nervosa (AN) (see further).[10] New insights from genetic engineering in mice, and clinical disorders, such as AN, where paradoxically fat mass is absent in the periphery but high in the marrow and is accompanied by an impairment in osteogenesis, have allowed investigators to examine the physiologic mechanisms that link bone mass acquisition and maintenance to energy utilization and adipose remodeling.

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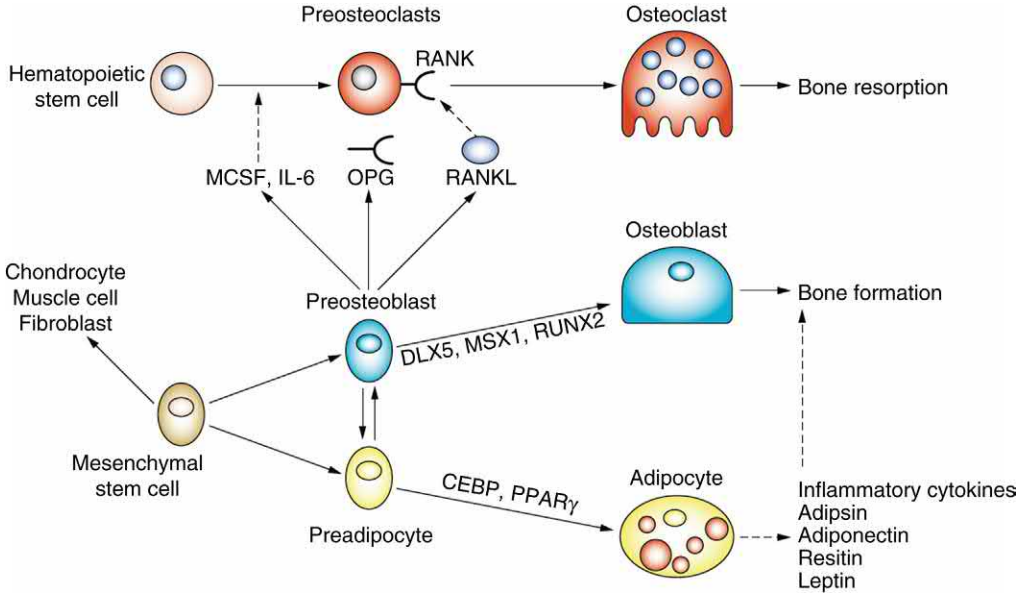

FIGURE 18.1 Cell fate decisions in the bone marrow are dependent on transcriptional factors for bone and fat, as well as energy needs in the niche.

On the other hand, only recently has attention focused on genetic determinants of bone and fat in humans using GWAS as a platform to explore that relationship. In this chapter we will focus on the underlying physiology of metabolic homeostasis as it relates to adipose tissues and the skeleton, as well as progress in defining common genetic determinants and their influence on those regulatory pathways.[11]

2 THE COMPLEX RELATIONSHIP OF ADIPOSE TISSUE TO BONE MASS

Body composition includes the amount and distribution of bone, adipose tissue, and water. Adipose tissue is generally considered within the context of three types of fat, defined by location, function, and morphology: brown adipose tissue (BAT) which is thermogenic and generally found in the interscapular region from birth; WAT, which is the storage depot for triglycerides and fatty acids and is found in visceral, perirenal, and subcutaneous depots; and beige or brown-like adipose tissue, found in some subcutaneous tissue and regulated by sympathetic tone.[12,13] Importantly, the relationship of bone mass to bone remodeling and skeletal density is dependent on the type of adipose tissue, hence one has to be cautious in interpreting the relationship of weight alone, or BMI alone to bone mass or skeletal fragility. Similarly, there are likely to be shared genetic determinants of bone and fat that differs by the type of adipose tissue being considered.

Excess WAT with inflammatory characteristics defines the obesity-metabolic syndrome of accelerated atherogenesis, glucose intolerance, and high serum lipids. Increases in WAT volume in mammals arise most commonly from expansion of adipocytes rather than the recruitment of new preadipocytes, although it has been suggested that approximately 10% of adipocytes in peripheral depots arise de novo per year. There is some debate about the source of brown-like adipose tissue and whether transdifferentiation between white and beige can occur. As such the effects of obesity on bone health are complex; increased mechanical loading due to greater body weight generally has a positive impact on bone, due to adaptations from greater loading on the skeleton while proinflammatory cytokines and adipokines in visceral adipose tissue (VAT) may have a negative impact, particularly on trabecular bone mass. The relationship of BAT to bone mass remains unclear with several lines of evidence to suggest there may be a positive effect, although these data are from association studies.

The interactions between BMI, BMD, and fracture risk have been extensively studied. A metaanalysis of 12 prospective studies involving almost 60,000 individuals by Delaet and coworkers found that increased BMI was associated with a reduced risk of fracture in men and women.[14,15] However, after adjustment for BMD, only the association with decreased hip fractures remained significant. This finding suggested that the positive effect of BMI on fractures is largely mediated through higher BMD, while the residual protective effect of high BMI on hip fractures may be related to the shock-absorbing effect of increased adipose tissue over the greater trochanter. A second metaanalysis reported similar protective effects of obesity on hip fracture.[16] Importantly, recent evidence indicates that overweight and obese individuals, while

being protected from some fractures, are at *increased* risk for ankle, humerus, and vertebral fractures.[17]

Previous assessments of adiposity have relied mainly on BMI or DXA assessments of total body fat, which unlike computed tomography (CT), cannot distinguish subcutaneous adipose tissue (SAT) from VAT. This may be relevant, as these two adipose tissue types have distinctive characteristics in terms of cytokine and adipokine production, insulin sensitivity, thermogenesis, and relationships to bone metabolism. Notably, VAT has a stronger association with metabolic syndrome and T2D than SAT.[18] Growing evidence indicates a negative association between VAT and areal BMD.[19,20] The negative effects of VAT on bone metabolism may be mediated by VAT-associated chronic inflammation and activation of proinflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) which induce bone resorption by stimulating osteoclast activity through upregulation of the RANKL/RANK/OPG pathway.[21] Visceral obesity may also affect bone turnover and metabolism through over- and undersecretion, respectively, of adipocyte-derived leptin and adiponectin.[22] On the other hand in a large cross sectional study of extreme microCT in young and older thin and obese individuals, both trabecular and cortical bone mass was higher in the obese subjects.[23] In addition to the factors noted aforementioned, obesity and T2D have been associated with a greater volume of marrow adiposity, which in turn could contribute to enhanced skeletal fragility.[24] These findings raise the intriguing possibility that obesity has differential effects on the skeleton depending on adipose type, depot, and distribution.

There is considerable controversy regarding the direct effect of insulin on bone cells. While evidence from some rodent studies indicates that insulin stimulates osteoblast proliferation and increases histomorphometric indices of bone formation by two- to threefold,[25] recent in vitro studies report that insulin signaling in osteoblasts also promotes bone resorption.[26,27] Similarly, clinical studies across a wide range of hyperinsulinemic states, including polycystic ovary syndrome and lipodystrophy, feature the consistent finding of high BMD.[28] The most direct evidence of the effect of hyperinsulinemia independent of fat mass comes from a study by Abrahamsen and coworkers who measured the association between BMD and insulin sensitivity index following an oral glucose tolerance test.[29] They reported that insulin sensitivity was negatively correlated with total aBMD independent of BMI and that the influence of fat mass on BMD disappeared after adjustment for insulin sensitivity. These data suggest that the effects of the insulin receptor on bone are independent of BMI. In light of the evidence, it would appear that in the early stages of T2D, the detrimental effects on the skeleton due to the metabolic dysregulation of obesity are probably counteracted to a large extent, by the hyperinsulinemia secondary to insulin resistance (in the absence of insulin resistance in skeletal tissues), and to a lesser degree, by the mechanical effects of increased body weight associated with obesity.

The relationship between BAT and bone mass has been much less extensively studied, and is only starting to be investigated as better imaging techniques have defined the volume of BAT by PET/CT. In several reports, the amount of BAT in young and early adult women and men has been positively associated with areal BMD.[30,31] However, when considering these correlations, muscle mass must be considered since there is a strong relationship between BAT and overall muscle volume, likely due to the expression of the common transcription factor Myf5 in both tissues. The relationship between brown-like or beige adipose tissue and bone has also been poorly defined. However, subcutaneous fat tissue has generally been positively related to bone mass in both adolescent and adult individuals, but whether this is related to gravitational forces on the bone, or due to secretory factors is not known. Interestingly, Lecka-Czernik and coworkers have shown that, when beige fat is enhanced by transgenic over expression of the transcription factor, FoxC2, bone mass is increased.[32] Hence, it is apparent that multiple factors determine the relationship of fat to bone.

How do these findings relate clinically to fracture risk and importantly to potential genetic determinants of fracture? As noted, obesity has long been thought to be protective for the skeleton, while low body weight, particularly in elders, is still considered a major risk factor for fractures. In the 10-year fracture risk algorithm (FRAX), now widely used in clinical practice, body mass index (BMI) can be substituted for bone mineral density (BMD), albeit imperfectly, to estimate absolute fracture risk. This relationship can be attributed to greater skeletal protection from falls, excess loading on the skeleton, and the extraovarian contribution of estradiol from aromatase activity in fat tissue. On the other hand, osteoporosis and obesity can coexist. For example, syndromes of fat redistribution, such as Cushing's disease and the drug induced lipodystrophies are associated with low rather than high bone mass.[33] Type II diabetes mellitus, despite the obesity, is actually associated with a greater number of fractures, and thiazolidinediones, highly effective drugs for diabetes, have been reported to increase the risk of peripheral fractures.[34] Furthermore, the extent of fat deposition in vertebral bone marrow correlates negatively with bone mass and indicates a greater fracture propensity.[35] Interestingly, in some cohorts, the percent of total fat mass, when body weight is kept constant, is strongly, but inversely associated with BMD.[36] These findings raise the question of whether fat as an endocrine organ, may exert distant but detrimental effects not only on the vasculature, but also on the skeleton. Finally, a disturbing

picture has emerged from the ongoing obesity epidemic. The prevalence of radial fractures in young adults has increased dramatically over the last decade, and one of the strongest predictors of fracture in this population is excess body weight.[37] The mechanism(s) responsible for this relationship is unknown but is likely to be multifactorial and related to life style, nutrition, and genetic determinants, as well as the load exerted on the upper limb in a fall. To summarize, the complex relationship of obesity to bone mass requires much further delineation and this in turn complicates genetic studies of common determinants for these phenotypes. New GWAS studies for fracture and body composition are currently being analyzed and may provide more insights.

3 A COMMON ORIGIN FOR FAT AND BONE CELLS

Bone marrow surrounds trabecular elements in the skeleton and is composed of multiple cells that are pluripotent.[38] In addition to the hematopoietic elements that include red and white blood cells, platelets, and their progenitors, the bone marrow stroma or connective tissue, contains mesenchymal stromal cells, an adult stem cell population capable of extensive self-renewal and plasticity.[39] Stromal cells are regulated by endocrine, paracrine, and autocrine signals, and in response can enter bone, cartilage, or fat lineages, depending upon the activation of intrinsic transcription factors.[40] The allocation of stem cells into bone forming osteoblasts, some which may enter the circulation, is accelerated after skeletal injury, as well as during the rapid growth phase of puberty and with administration of parathyroid hormone 1–34.[41,42] Runx2/Cbfa1 and osterix are two of several bone-specific transcription factors that are required for this process (Fig. 18.1).[43] In contrast, entry of stromal cells into the fat lineage occurs through activation of the nuclear receptor PPAR-γ2 by endogenous fatty acids or exogenous ligands (Fig. 18.1).[13] Stem cell specification into the fat or bone pathway is often considered as an either-or-paradigm; that is, commitment of these cells is exclusive to one lineage or the other, based principally on PPAR-γ2 complex activation by a class of drugs called the thiazolidinediones (TZDs: rosiglitazone or pioglitazone).[34,44] These agents improve insulin sensitivity and enhance bone resorption while shifting mesenchymal cell fate from preosteoblasts to preadipocytes.[45] The clinical outcome of treatment with the TZDs is often manifested as improved glucose tolerance, associated with modest weight gain and increased marrow adiposity at the expense of skeletal mass.[46] However, in other circumstances, enhanced marrow adipogenesis can coexist with active bone formation, such as the inbred mouse strain, C3H/HeJ, or after treatment with certain PPARG agonists.[45,47] Along these lines, emerging evidence suggests there may be a mixed population of cells that have transcriptional characteristics found in both fat and bone cells. Westendorf and coworkers have noted that some Runx2 positive cells have large perilipin positive lipid droplets in HDAC3 null mice consistent with the hypothesis that these cells are "osteo-adipocytes."[48] Lanske and coworkers have shown that some marrow adipocytes express RANKL, a stromal cell cytokine characteristic of preosteoblasts.[49] Hence, the progression or "switch" of stem cells into either the fat or bone lineage may not be mutually exclusive, and supporting the concept of plasticity during fate decisions. Moreover, genetic determinants are likely to be important at this critical junction for both physiologic and pathologic states (see later).

Although much of what we have learned about the "switching mechanism" in mesenchymal stromal cells has come from basic investigations, clinical experiences have also been very illustrative. For example, glucocorticoids are remarkable for their ability to enhance marrow adipogenesis at the expense of osteoblast differentiation.[50] In the syndrome of glucocorticoid-induced osteoporosis, bone loss is quite rapid at a time when fat mass is enhanced, particularly in central depots. Similarly, the TZDs reduce bone mass, and increase the risk of peripheral fractures possibly because of the switch in stem cell allocation into the adipocyte lineage.[51] Another less well known example of "switching" occurs with aging. Fatty infiltration of the vertebral marrow is a characteristic feature of older individuals, and can be recognized on MRI exams, usually as an incidental finding. Its presence, however, is inversely related to BMD and skeletal integrity in many circumstances.[52] Importantly, cell fate decisions are also related to their metabolic program (see later), which is tied to energy availability and illustrated by the syndrome of AN. Thus, bone and fat cells share a common origin, and their fates are interwoven in a context specific manner. Pharmacological manipulation of mesenchymal stem cells to reduce adipogenesis or enhance osteogenesis is a promising new but yet untested avenue for therapeutic investigations.

4 BIOENERGETICS OF CELLS IN THE BONE MARROW NICHE IN RELATION TO ENERGY NEEDS AND WHOLE BODY METABOLISM

It is likely that cell fate in the niche relates to metabolic flexibility and programming of progenitor cells. Each of the cells in the bone marrow niche has its own specific nutrient requirement in order to survive, particularly in a hypoxic environment linked to ATP demands. In general, the more differentiated the cell, the greater the energy needs. However, within that context, there

are major differences between mature osteoblasts and adipocytes. First, when considering survival and maintenance of MSC stemness in the relative hypoxia of the marrow, there is a need for metabolic adaptation. Stemness allows a stable pool of progenitors that can be called on in numerous circumstances, particularly injury and inflammatory states [53] Hypoxia induces the stabilization of HIF1α, a major transcription factor for stem cells and progenitors, as well as multiple downstream target genes, particularly VEGFα.[54]

Metabolic reprogramming of quiescent cells is necessary to prevent differentiation and this occurs through a shift from oxidative phosphorylation to glycolysis. Importantly, glycolysis, although less efficient in generating ATP than mitochondrial oxidation, reduces oxidative stress and reactive oxygen species (ROS) generation, key elements that drive stem cell differentiation. Although glycolysis is the major driver of ATP generation in MSCs, entrance into a specific differentiation program, either adipogenic or osteogenic, requires distinct metabolic requirements that are very context specific.[55] For adipocytic differentiation, several studies have suggested that mitochondrial oxidation of fatty acids and the generation of ROS are essential to achieve full maturation. The process of glucose entry and fatty acid oxidation through the Krebs Cycle generates more molecules of ATP per mol of glucose (36:1) than glycolysis (2:1), but it comes at a cost as mitochondrial respiration leads to the generation of ROS from the electron transport chain (ETC). ROS (e.g., H_2O_2, superoxides) can further suppress mitochondrial respiration and promote an adipogenic program that is associated with more insulin resistance and less lipolysis.[56,57] Excess ROS in adipocytes may also cause mitochondrial DNA damage or further changes to complex I in the ETC leading to metabolic dysfunction.[58] Although some ROS is generated during normal adipocyte differentiation; much less is produced during early stages of osteoblast maturation.

During osteoblastic differentiation, glycolysis is the predominant ATP generator, even though it is less efficient per mol of glucose.[59] Glycolysis can occur rapidly and can happen in both hypoxic and normoxic states (i.e., Warburg effect). Two key proteins, Glut1, the principal glucose transporter and lactate dehydrogenase (LDH), the enzyme that converts pyruvate to lactate are essential during glycolysis. Karsenty and coworkers showed that in osteoblasts, glucose uptake through the Glut 1 transporter inhibits AMPK, which in turn prevents ubiquitination of runt-related transcription factor 2 (Runx2).[43] In a feed-forward system, Runx2 begins the differentiation program in osteoblasts and increases Glut1 expression. Furthermore, preosteoblasts differentiate under the influence of various ligands, particularly the Wnts and insulin-like growth factors (IGFs). Long and coworkers demonstrated that glycolysis is a major feature of Wnt3a induced osteoblast differentiation.[60] More recently the same group reported that HIF1α which is important for stemness, is also a critical transcriptional regulator of glycolysis, triggered in part, by relative hypoxia in the bone marrow niche.[61] Remarkably, much older ex vivo studies from Neuman and Nichols demonstrated that parathyroid hormone (PTH) treatment produced lactic acid in calvarial osteoblasts supporting the tenet that osteoblasts utilize glycolysis to generate lactate and fuel collagen synthesis and mineralization.[62] Guntur et al. also showed that glycolysis was essential for terminal differentiation of osteoblasts and that oxidative phosphorylation was more important early in the differentiation scheme.[63] Thus, it is likely there is a distinct metabolic program that features a transient phase of oxidative phosphorylation following glycolysis and that is then switched off as glycolysis reemerges as a predominant driver of ATP. Indeed, recent studies suggest that both oxidative phosphorylation and glycolysis are occurring in differentiating cells, and the relative proportion that contributes to ATP determines the final energy production. Too much oxidative phosphorylation or glycolysis can inhibit the other, so there clearly must be a fine context specific balance.

The transient phase of mitochondrial respiration is time sensitive and may occur in vitro between days 3 and 9 of osteoblastic differentiation. During this time period, AMPK is activated and this may induce lipophagy, as well as oxidative phosphorylation.[64] Other studies have shown that metformin, which upregulates AMPK can enhance differentiation but only during specific time periods. In a similar vein, Karner et al. demonstrated that glutaminolysis is also essential for osteoblast differentiation through the Wnt signaling system.[60] Hence, there are at least three substrates for ATP generation and differentiation of MSCs: glutamine, which enters the Krebs Cycle via alpha ketoglutarate; glucose, which through glycolysis can generate lactate, as well as ribose nucleotides via the pentose phosphate shunt; and fatty acids, which are metabolized via acetyl coenzyme A (CoA) in the mitochondria. Generation of acetyl CoA can also lead to enhanced nuclear acetylation that in turn can impact transcriptional processes unifying the processes of protein production and energy utilization. Finally, autophagy cannot be overlooked as a fueling mechanism for the cell particularly during times of stress or nutrient deficiency. AMPK stimulates autophagy, as well as glycolysis and inhibits mammalian target of rapamycin (mTOR) and overall protein synthesis.[65] This would also lead to increased fatty acid entry into the mitochondria for ATP generation as lipophagy is also stimulated.

In summary, the bone marrow niche has significant energy requirements that are tissue and temporal specific. Allocation into the osteoblast or adipocyte lineage is dictated by multiple transcription factors, which in

turn must be governed by specific metabolic programs and their inherent flexibility. Changes in energy availability are certain to alter cell fate decisions and these in turn can impact metabolic homeostasis. Taken together, the determination of the identity of marrow adipocytes as osteoblasts in disguise, or a novel fat cell, is a critical question because of the intimate relationship between adipogenesis and skeletal remodeling, particularly in states of energy insufficiency. Genetic determinants of metabolic programs are certain to link bone and fat metabolism.

5 CONTROL OF SKELETAL AND ADIPOSE TISSUE REMODELING

Bone and fat are functionally related through a complex neuroendocrine circuit that involves the brain, adipose depots, and the skeleton. In mammals, the sympathetic nervous system can drive lipolysis through release of norepinephrine, which activates beta adrenergic receptors of which there are three (β1–3 AR) in adipocytes. The effects of the SNS on bone are more complex (Fig. 18.2) and are driven primarily through activation of the β2AR. Interrelated to the neural modulation of bone and fat, several hormonal factors are also regulatory particularly during puberty. For example, during linear growth and the acquisition of peak bone mass, surges in growth hormone and gonadal steroid secretion provide a stimulus for skeletal expansion, and stem cell recruitment into cartilage and bone. These processes are fueled through lipolysis of WAT. As such, malnutrition or undernutrition can cause short stature and a severe reduction in peak bone-mass. Thus, when considering the regulation of fat and bone by systemic and local factors, it is helpful to classify these as peripheral or central mediators. The peripheral mediators are adipokines that act via the CNS to regulate sympathetic outflow to both fat and bone tissue. Central mediators arise from the CNS as either neural peptides or paracrine factors.

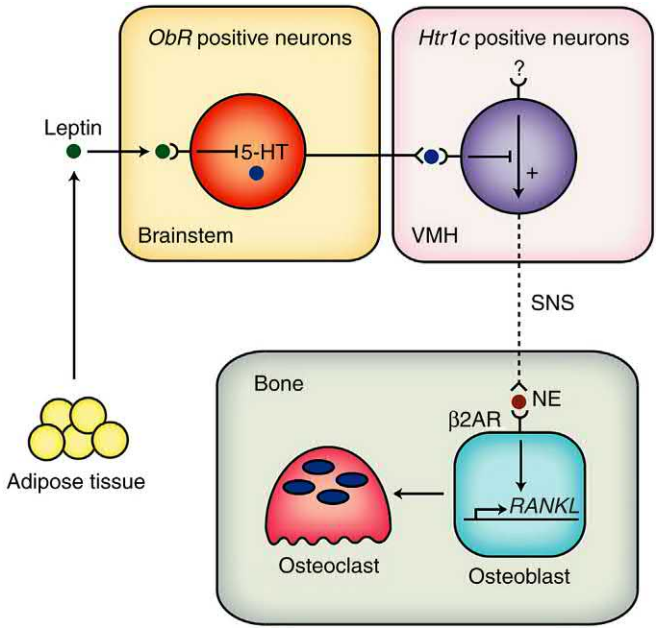

FIGURE 18.2 Adipokine regulation of bone mass via the brain stem and ventromedial hypothalamus and sympathetic nervous system (SNS).

5.1 Adipokine Regulation of Bone Remodeling

Leptin is an adipokine produced by fat cells.[66] The circulating levels of leptin are directly related to total fat mass. Leptin regulates appetite, reproduction, and energy utilization by crossing the blood–brain barrier and binding to a receptor in the hypothalamus. In the ventromedial nucleus of the hypothalamus, leptin triggers activation of the SNS. Deficiencies of, or resistance to leptin can cause obesity, impaired fertility, and changes in appetite, in both rodents and humans.[67,68] Surprisingly, the absence of leptin results in high bone mass, even though gonadal steroids are markedly suppressed in the animal model of total leptin deficiency, the ob/ob mouse. Studies of these mice demonstrated that the high bone mass of leptin deficiency is a result of reduced sympathetic tone innervating β2 adrenergic receptors in osteoblasts (Fig. 18.2). Those observations provided evidence that bone remodeling is regulated by a hypothalamic relay via the sympathetic nervous system, and is primed by leptin, the sensor of peripheral fuel status in fat depots. Not surprisingly, the system is more complex than originally perceived, and includes other hypothalamic networks, such as the neuropeptide CART, melanocortin 4 receptors, the neuropeptide Y receptor system, and neuromedin U.[69,70] Cannibinoid receptors that regulate appetite and energy balance also modulate bone turnover centrally and peripherally, principally by blocking sympathetic innervation. Karsenty et al. have reported that the bone specific protein, osteocalcin, when undercarboxylated regulates insulin secretion, gonadal status, and muscle activity.[71] If validated in humans, this connection provides another step in a complex integrative circuit that regulates body composition, although in this case, the master regulator is the skeleton.

Adiponectin is a secretory peptide made by differentiated adipocytes, primarily from WAT. Its expression occurs late in adipocyte differentiation and is regulated by the master transcription factor PPARG. Adiponectin can induce insulin sensitivity and is thought to be a modulator of glucose transport. It is upregulated by the thiazolidinedione class of antidiabetic agents. Recently, Cawthorn and coworkers demonstrated that adiponectin secretion also occurs in marrow adipose tissue (MAT) and this may regulate glucose levels in AN; a condition

associated with low-energy intake but high insulin sensitivity.[72] In experimental animals, adiponectin has been shown to have dual effects on bone mass; direct and indirect. In respect to the former, adiponectin has been shown to inhibit osteoblast differentiation in the bone marrow; on the other hand, adiponectin can downregulate sympathetic tone, thereby indirectly enhancing bone mass. Karsenty and coworkers have suggested that leptin and adiponectin act in a yin and yang mode in the CNS.[73] Hence, it is apparent from experimental evidence that one locus of control over body composition is modulated through the sympathetic nervous system.

5.2 Sympathetic Nervous System Control Over Fat and Bone Remodeling

Several lines of evidence point toward an efferent sympathetic pathway from the brain-controlling skeletal metabolism. Genetically engineered mice with both a global and conditional deletion of the β2 adrenergic receptor (B2AR) have high bone mass at 8 and 16 weeks of age. Mice treated with isoproterenol, a β-adrenergic receptor agonist, lose bone mass while treatment with the β-adrenergic receptor antagonist propranolol may protect against OVX induced bone loss.[5,74,75] Patients with reflex sympathetic dystrophy, a disease characterized by high sympathetic tone are prone to low bone mass, that at least in some cases can be mitigated by β-blockers.[76] Sympathetic overactivity has also been proposed as a contributing mechanism for microgravity induced bone loss during space flight although long-term studies have not been done.[77]

The link between leptin and bone via a sympathetic efferent was established by Efleritou et al., through failure to reverse the high bone mass phenotype in β2 adrenergic receptor (Adrβ2) deficient mice following ICV infusion of leptin.[78] The expression of Adrβ2 in osteoblasts provided another link in the pathway between hypothalamus and osteoblast, and mice with selective knockout of Adrβ2 on osteoblasts have a high bone mass from increased bone formation and reduced bone resorption (Fig. 18.2). Studies also indicated that sympathetic signaling in osteoblasts is responsible for regulatory control of osteoblast function through inhibition of osteoblast proliferation via circadian clock genes and that the sympathetic nervous system also favors bone resorption by increasing the expression of RANKL.[79–81] The balance of bone formation and resorption from chronic stress induced sympathetic activity may shift to favor bone resorption as seen with chronic stimulation of β-AR with low-dose agonist treatment in mice which induces bone loss mainly via enhanced bone resorption [79] suggesting the control of each cell type by the SNS is temporal. Second generation antipsychotics, such as risperidone, also upregulate sympathetic tone and uncouple remodeling; these effects can also be blocked with propranolol.[82]

6 ANOREXIA NERVOSA: ENERGY, BONE, AND ADIPOSE DEFICIENCIES

In humans, abnormities in fat metabolism associated with clinical states of extreme under- and overnutrition provide a global consideration of the relationship between fat, bone, and energy needs. Congenital lipodystrophies provide some insights, but are rare. The most common and yet most extreme and well studied disorder of low body fat and its effects on bone mass is AN, a primary psychiatric disorder affecting up to 1% of young women. AN is characterized by self-induced starvation, severe decreases in body adipose depots, and muscle mass, functional hypogonadotropic hypogonadism and substrate shunting to preserve vital functions.[83] Paradoxically, bone MAT is markedly enhanced and is now an additional hallmark of the bone component in this disease. Additional physiologic adaptations to starvation that impact bone metabolism and fat mass include hypercortisolemia and growth hormone resistance. Rapid and significant bone loss in adults and inadequate acquisition of bone during the peripubertal period in children is a hallmark of the disorder. In a community based outpatient study of 130 young women with AN, 92% of women were osteopenic and 38% met criteria for osteoporosis.[84] Bone formation is markedly reduced while bone resorption is increased in this disorder, and as noted, MAT is increased.[85] Insulin sensitivity is another metabolic feature of this condition, and in extreme cases can lead to severe hypoglycemia and death. Weight gain causes increases in bone formation, decreases in bone resorption and loss of MAT.[86,87] Estrogen replacement induces a modest reversal of the skeletal and metabolic phenotypes.[88,89]

Several hormone mediators have been linked to low bone mass in AN and estrogen is the most dominant. Its effects on bone relate to its known inhibition of osteoclast-mediated bone resorption. Intriguingly, loss of estrogen during the menopause also leads to bone loss but not to the same degree as in AN, in part presumably because of the timing of such loss. In addition, menopausal estrogen loss is often associated with increased visceral adiposity, which is generally not seen in the setting of chronic AN. Loss of estrogen in AN is due to hypothalamic changes as undernutrition suppresses GnRH pulsatility resulting in hypogonadotropic hypogonadism. However, the increased severity of bone loss in this disorder compared to that seen in other young amenorrheic women, as well as the failure of estrogen replacement to fully improve bone mass implicates other nutritionally dependent factors. For example, deficiencies in ovarian

androgens, hypercortisolemia, and very low serum IGF-1 levels, the latter a nutritionally mediated endogenous bone anabolic factor, may contribute to bone loss.[90]

The strongest predictor of increased bone mass in AN is weight recovery.[10] In a longitudinal study of women with AN, a 0.6% increase in hip bone mass was seen with weight gain. However, return of ovarian function is also a key factor as greater improvement in bone mass is seen in women who regain both weight and menses. Biochemical markers of bone formation increase with weight gain but decreases in bone resorption markers occur only in those women who regain menses and have adequate endogenous production of estrogen.[91]

Hypercortisolemia, thought to be due to activation of the CRH–ACTH–adrenal axis, occurs in AN and elevated cortisol secretion, as well as loss of diurnal rhythmicity.[92] Because hypercortisolemia is known to suppress bone formation and increase marrow adiposity, this effect may contribute to low bone mass in patients with AN.[93] Despite cortisol excess, central adiposity has not been seen, presumably due to the generalized decrease in fat mass. In children with AN, baseline cortisol levels predicted increases in body fat with disease recovery.[94] With weight recovery in adults, truncal fat is also disproportionably increased, hypothesized to occur because of new substrate availability. In such patients, assessment of body composition and fat distribution by anthropometry, dual-energy X-ray absorptiometry, and whole-body MRI demonstrated that waist-to-hip ratios (WHR), total truncal, visceral, and intramuscular adipose tissue were significantly greater than in healthy controls of comparable weight.[95] AN is also characterized by GH resistance with elevated GH levels but decreased hepatic production of IGF-1.[96] There is a negative association between GH secretion, BMI and fat mass. Because GH directly and indirectly through IGF-1, increases bone mass, the lack of GH action also contributes to the very low BMD in this disorder, and possibly, worsens the increase in MAT.

The development of AN during adolescence is of particular concern as pubertal delay may occur.[92] Since adolescence is a time of rapid bone accretion and pubertal changes in body composition, the onset of disease during this critical window in development can dramatically affect peak bone mass accrual with consequences that may endure throughout life. The development of AN in childhood can lead to a reduction in bone size and volumetric BMD. Of note, the site and severity of specific deficits in both these parameters showed a maturational dependency on the age of onset of AN, as well as disease duration.[97] In sum, the skeletal components of this disease are directly related to changes in body composition and energy metabolism and the timing of the disease onset.

Adipokines play a major role in mediating changes in bone and fat mass. As noted previously leptin is an anorexigenic hormone. Anorexigenic and orexigenic hormones impact food regulation, energy expenditure, and bone mass, and vary with body fat. Although weight loss typically precedes amenorrhea in AN, an interesting observation has been that reproductive function can be maintained in some women, despite very low weight.[98] This may be due to several factors, including fat mass and leptin levels. Low-percent body fat and decreases in adipokine secretion, specifically leptin, may be a key mediator of reproductive function and likely impacts bone mass.[99] Although low in both groups, lumbar BMD is higher in eumenorrheic than amenorrheic women with AN, despite similar weight.[92] Of importance, mean percent body fat, total body fat mass, and truncal fat were higher in eumenorrheic than amenorrheic patients. This increase in body fat was associated with higher leptin levels in the eumenorrheic women than the amenorrheic women. Indeed, fat mass emerges as an independent predictor of bone density in regression models, including other variables, such as multiple body composition and gonadal function.[92]

Adiponectin also plays a role in the pathophysiology of the metabolic components of AN. Most but not all studies have found high adiponectin levels with AN.[100] In women with the restrictive subtype of AN, BMI, body fat content, and serum leptin levels were significantly decreased. Of note, an inverse relationship was found between adiponectin levels and BMI such that the elevated adiponectin levels compared to normal controls has been suggested to reflect the marked decrease in body fat in AN.[101] In a study of adolescents with AN, Misra et al. found that adiponectin levels were significantly associated with bone density of the lumbar spine and femoral neck.[102] Although the mechanisms whereby adiponectin might affect bone mass are unclear, changes in fat mass and insulin sensitivity in AN clearly play a central role. In a study,[103] plasma adiponectin levels were elevated in young women with AN compared to controls in the setting of reduced fat mass and impaired insulin-stimulated glucose disposal. Therefore, markedly reduced fat mass increases adiponectin secretion that may then affect bone mass directly or through changes in insulin sensitivity. Cawthorn et al. also demonstrated that high marrow adiposity in anorexia was related to increased serum adiponectin, suggesting this might be the origin of insulin sensitivity in this syndrome.[72] In sum, multiple changes in adipokines contribute to the pathogenesis of low bone mass in patients with AN.

Other appetite regulating hormones that may mediate bone loss in AN include peptide YY (PYY), an anorexigenic hormone, and ghrelin, which is orexigenic. Produced in the intestinal L cells in response to caloric intake, PYY acts via the Y2 receptor, which when deleted

in the hypothalamus, causes increased bone formation in rodents.[104,105] Obesity is associated with low-levels of PYY and these correlate inversely with BMI.[106] PYY levels have been reported to be elevated in AN compared to lean or obese controls.[107] In a study of girls with AN, PYY levels were higher compared to controls and PYY predictors included BMI, fat mass and resting energy expenditure.[10] In addition to the inverse association between fat mass and PYY levels, there was a negative association between fasting PYY levels and fat intake. In that study, PYY levels predicted markers of low bone turnover consistent with hypothesized effects of PYY increases on bone metabolism. This finding is also consistent with the enhanced osteoblastic activity noted in Y2 receptor knock-out studies. In adults with AN, mean overnight PYY levels were strongly and inversely correlated with BMD at the spine, total hip, femoral neck, and radius and, in association analysis, PYY was the primary determinant of spine BMD.[108]

Another appetite hormone linked to both bone and fat in AN is ghrelin. The orexigenic stomach-derived peptide hormone ghrelin is secreted in response to fasting, peaks before meals, and stimulates secretion of both growth hormone and ACTH. Ghrelin levels have been consistently reported to be elevated in AN and positive correlations are found between the ghrelin/obestatin, (i.e., proteins derived from the same gene but act as antagonists), ratio, and BMI.[109,110] Ghrelin also has effects on adipogenesis. Thompson et al. infused ghrelin, des-octanoyl ghrelin, or synthetic GHS-R_{1a} agonists into rats with varying severity GH deficiency of and demonstrated that a major circulating form of ghrelin, des-octanoyl ghrelin, has a direct adipogenic effect.[111] Ghrelin administration increases osteoblast proliferation.[112] In a prospective observational study of adolescents with AN, ghrelin was an important predictor of changes in spine and whole-body bone mass.[113]

The pathophysiologic changes from AN highlight the intimate relationship between fat mass, energy needs, and BMD. Although not a genetic disease, this syndrome has illustrated how complex those interactions are in humans and animal models.

7 GENETICS OF FAT AND BONE IN ANIMAL MODELS

As demonstrated in the previous discussions, fat and bone are intimately linked and their health are a part of the balance inherent in whole body metabolic homeostasis. To assess the genetic and gene × environmental determinants of this interaction, animal models have been a major investigational tool. There are two main categories of studies for identifying association between genes and phenotypes. The first is the reverse genetic study where the starting point is a gene and the goal is to ascertain what and how phenotypes are impacted. The second is a forward genetics study where the starting point is a phenotype or phenotypes and the goal is to uncover the genetic etiology. A historical workhorse of forward genetic mapping in animal models has been the two-strain intercross, with the output of these studies being quantitative trait loci (QTL) or regions of the genome containing polymorphic differences associated with the trait of interest. Given that fat is an important part of meat quality many studies genetically linking fat mass and bone size have been conducted in species of agricultural importance. Most of these studies have looked at fat weight as comapping with bone weight or bone area, which are not traits that we are primarily interested in for understanding bone disease.[114–116] One study in sheep did demonstrate comapping loci on ovine chromosome (Chr) 24 for total carcass fat mass and bone density as measured by CT.[117] Like many QTL mapping studies, these studies suffered from poor mapping resolution, so while these studies can suggest pleiotropy, the possibility of comapping cannot be ruled out.

This issue of comapping between obesity and bone QTL has been explored in greater depth in congenic mouse studies. A congenic is a strain with part of a Chr moved from one genetic background to another. These strains are generated by selective breeding. The resulting animals allow confirmation or de novo establishment that genetic regions contain polymorphism(s) impacting phenotype and allow study of the biology associated with a QTL without necessarily knowing the causative gene. Using a congenic strain wherein C3H/HeJ (C3H) alleles for middistal Chr 6 were introgressed on to a C57BL/6J (B6) background, we showed that there was a locus simultaneously impacting obesity, bone mass, and serum IGF-1 levels. Upon further study, we also showed that this locus impacted marrow fat levels, as well as osteoblast lineage allocations.[118] Using consomic strains, which are akin to congenics, but differ in that a whole Chr has been transferred instead of a region (see reference later), Reed et al., determined that genes on mouse Chr 7 also impact fat mass and bone mass.[119] Using a combination of the F2 intercross strategy and the congenic mouse approach, Jerez-Timaure et al. were able to narrow and partition a wide QTL (38 cM in span) previously associated with fat mass and with BMD on distal mouse Chr 2.[120] Specifically, they used a recombinant progeny approach to narrow this locus down for each phenotype of interest. Thus, they demonstrated that there is actually a cluster of genes impacting various combinations of body composition traits underlying it. The phenotype of BMD comapped with actual fat mass and with percent lean mass (so called leanness) but adiposity (percent total body fat) independent of body size was actually controlled by a separate and slightly

distal genetic region. As the BMD phenotype mapped in this study is whole body BMD as measured by DXA, it is difficult to separate this phenotype from body size.[121] This study is very important as it highlights dangers of declaring pleiotropy for "bone" from "weight" and "adiposity" QTL, despite the obvious physiology that would support, such a hypothesis.

Adiposity and obesity, are well understood to be influenced by environmental factors including factors, such as diet. In a very interesting study of inbred mice fed either a low fat chow diet or high fat–arthrogenic diet, Li and coworkers used structure equation modeling to investigate the link between BMD and fat mass.[122] They concluded that there was a genetic link between these two phenotypes, but the relationship was modified by dietary fat challenge. Specifically, their study suggested that both fat mass and lean mass have direct effects on BMD when mice are fed a high fat diet, but only lean mass, not fat mass has an effect when mice are fed a normal chow diet. While this study was not designed to identify individual genes and loci impacting this relationship, it is an interesting study that highlights that environment influences this fat-bone dynamic.

To address this issue more directly, Leamy et al. mapped QTL for a six skeletal traits in an advanced intercross population of mice made by interbreeding C57BL/6J mice and a strain selected for voluntary high running on a cage wheel.[123] An advanced intercross population or line is a population of animals or plants that has been interbred for more generations than the standard, two-generation F2 intercross. The advantage of an advanced intercross population is that mapping resolution is improved, but the disadvantage is that they take a long time to make and are very costly to maintain.[124] In the Leamy study, the mice examined were at G10 of intercrossing and half of the mice were fed a high fat diet, with the remainder a low fat control diet. While many QTL for femoral, vertebral and whole body BMD were mapped in this study, none were found to interact with dietary fat. A locus on Chr 7 for femoral shape and one on Chr 1 for femoral length were found to interact with dietary fat such that these loci were suppressed by the normal chow diet.

7.1 Shared Genetic Determinants of Bone and Fat in Animal Models

There are no other direct studies of high-fat diet interacting QTL where high fat feeding and control diet were used in the same study. However, two B6 by C3H crosses have been described in the literature. In the first of these, nearly 1000 female F2 mice where used to map loci for volumetric femoral and vertebral BMD.[125] In this study all mice were fed a chow diet. In a second similar study, 145 female and 164 male F2 mice were fed a high fat diet for 14 weeks and loci were mapped for femoral areal BMD.[126] A head-to-head comparison of these two studies provides putative existence of many diet-interacting QTL. One of these QTL is on distal Chr 1. Follow up studies with congenic mice provide evidence that this locus does indeed interact with dietary fat and suggests *Aim2* and a previously uncharacterized gene, *Gm4955* (*AC084073.22)* as likely candidates.[127] Second of these loci is on Chr 6. This locus was discussed previously as one that controls BMD and adiposity, and we have also shown that it interacts with dietary fat. Bioinformatics narrowing of this locus suggested peroxisome proliferator activated receptor gamma (*Pparg*) as a likely candidate gene. We then studied this gene in humans and determined that in men and women, allelic variation in this gene impacted BMD in humans via an interaction with dietary fat.[127]

In summary, there is strong evidence to show a genetic link between obesity, adiposity, and bone. This link is heavily influenced by dietary factors and almost certainly by gut microbiota. Unfortunately, while there have been many loci showing comapping of fat and bone traits, making the determination of pleiotropy has been difficult. Much work still needs to be done to determine which what gene(s) underlie many of these loci and if the impact on bone is via actions in adipose tissue, directly in bone or both.

8 GENETICS OF FAT AND BONE IN HUMANS

Obesity and osteoporosis, and their related quantitative phenotypes, are highly heritable.[128,129] Due to this fact and the enormous burden of each to human health, significant emphasis has been placed on dissecting their genetic basis. The initial genetic studies of obesity and osteoporosis used linkage analysis in families and candidate gene association studies. In retrospect, these approaches were plagued by limitations and, relatively speaking, little was learned. However, the approaches used to understand the genetics of fat and bone in humans have evolved over the last 30 years.[130] This evolution has been driven by large consortia efforts to generate catalogs of human genetic variation [131,132] and technologies capable of genotyping millions of single nucleotide variants (SNVs) in thousands of individuals. These advances have led to the era of genome-wide association studies (GWASs).

GWASs have revolutionized our understanding of complex diseases and disease-associated quantitative traits in humans.[133] In a GWAS, the genotypes of millions of SNVs are evaluated for effects on a disease-related trait, such as BMD, or for a difference in the frequency of

SNV alleles between "disease" cases and controls.[134] The discovery of associated variants by GWAS highlights a genomic region (usually spanning 5–150 Kbp) that contains one or more potential "causal" variants impacting the function or regulation of one or more target genes. Associations can then be dissected to identify which variants and genes are truly causal. One of the advantages of GWAS is that it is an unbiased gene discovery approach. Additionally, the regions identified by GWAS are narrow, implicating a small number of genes, and resolution is only limited by the local extent of linkage disequilibrium.[134] Importantly, recent works suggests that therapeutics targeting genes identified by genetic means (typically GWAS) are twice as likely to make it through the drug development pipeline than drugs targeting genes not implicated by genetics.[135] This suggests that GWAS data will become an increasingly important source of new drug targets.

Traits related to both, obesity and osteoporosis have been interrogated by GWASs. For obesity, GWAS in cohorts with over 100,000 individuals have identified large numbers of loci for body fat percentage,[136] BMI,[15] and body fat distribution (WHR) and waist/hip circumference.[137] The largest GWAS for WHR ($N \sim 224$ K people) identified 49 loci associated with WHR adjusted for BMI and an additional 19 loci associated with waist and hip circumference measures.[137] In addition, the largest GWAS for BMI was performed in ~339 K people and identified 97 loci.[15] Several GWASs have been performed for bone phenotypes. Most notably, BMD has been the trait of choice for GWAS, primarily due to its high heritability ($h^2 > 0.50$) (P, association with fracture [138] and availability in large cohorts. The largest GWAS for BMD was conducted by GEnetic Factors for OSteoporosis Consortium (GEFOS) for lumbar spine and femoral neck BMD in ~32 K individuals (with replication of the most significant associations in another ~50 K people) and identified 64 independent associations.[139]

Several important observations have been made from obesity and BMD GWASs. First, it is clear that each is highly polygenic, meaning that hundreds, if not, thousands of variants are influencing each disease. Second, common variants have small and subtle influences on body composition. All of the significant variants identified in the BMI and BMD GWASs described previously in aggregate ~3% of the phenotypic variance for either phenotype. Third, most genetic variation impacting both phenotypes, identified by GWAS, is noncoding and likely impacting some aspects of gene regulation. However, the most exciting observations coming out of GWAS are that many loci implicate genes not previously demonstrated to be involved in fat or bone biology. This supports the premise that within the GWAS findings data is a tremendous amount of information on very new biology. The challenge we now face is identifying the genes responsible for GWAS loci and ultimately translating those genes into new therapeutics.

8.1 Shared Genetic Determinants of Fat and Bone in Humans

Body weight, and its component phenotypes, lean mass and fat mass, have been demonstrated to be positively associated with BMD. However, the exact role of fat, and its interaction with bone, appears to be much more complicated. While increased fat mass has been shown to provide protection against fractures, recent studies indicate that, increased weight is protective against fractures, but fat mass per se has a negative impact on bone.[140] It is thought that the protective effects of increased weight are due to amplified mechanical forces, which cause bone to adapt by increasing its strength (through increased BMD or bone size). In contrast, adipose tissue itself may have deleterious effects on bone. This latter idea has come from observations that the relationship between fat mass and BMD is negative after adjusting for the effects of body weight (i.e., in individuals with the same body weight, those with higher percent fat mass have lower BMD.[140]

One of the key unanswered questions is what mediates the relationship between fat and bone, the environment or genetics or both? Studies have estimated the environmental and genetic correlations (the extent that the same environments or shared genes are responsible for the phenotypic relationships) between fat and bone.[141] In a study of monozygotic and dizygotic twins Nguyen et al., studied the relationships between lean mass, fat mass, and BMD.[141] They found that most of the association between fat mass and BMD were due to a strong environmental correlation, with a small nonsignificant genetic correlation. However, Zhao et al., demonstrated that after adjusting BMD for the effects of body weight, there was a significant negative genetic correlation between fat mass and BMD.[140] These observations suggest that the positive effects of obesity are mainly driven by environmental factors, whereas the negative impact of adipose tissue independent of body weight are determined, in part, by a common set of genes. Furthermore, the Zhao et al. study demonstrated that shared genes with a positive impact on fat, negatively impacted bone and vice versa.[140] It should be noted that very recent studies which use GWAS results to estimate genetic correlations between traits have shown small, but significant, positive genetic correlations between BMD and BMI and waist-to-hip ratio. This is interesting since the BMD GWAS data used weight-adjusted BMD.

Although these studies suggest that there should be shared sets of genes influencing both, fat and bone, the

results of GWAS to date have not been able to uncover such genes. Pickrell et al. used GWAS data to seek genomic regions harboring associations with more than one disease or trait. Included in their analysis were the GWAS results described previously for waist-to-hip ratio, BMI and BMD.[142] They found no such regions for lumbar spine BMD and obesity phenotypes but one region on Chr 12 (53–54.8 Mbp) linked to femoral neck BMD and WHR. SNVs in the latter region had a positive impact on femoral neck BMD and a negative impact on WHR, consistent with the negative genetic correlations between fat and bone described previously. In a separate study, Liu et al. performed a bivariate GWAS for BMI and BMD.[143] Though none of the variants typed in the study reached genome-wide significance, the top two SNPs were located within introns of the SRY-box 6 (*SOX6*) gene which has been implicated in bone and cartilage development, as a possible contributor to the fat and bone relationship.[144]

In summary, for humans, the genetic relationship between fat and bone appears to be more complex than anticipated and additional studies will be needed to clarify that interaction. However, there is a very strong rationale for pursuing this in depth. Not only could identification of genetic loci lead to specific molecular targeting and the potential for novel therapies for obesity and osteoporosis, but such discoveries could also have profound implications for our understanding of whole body metabolism.

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CHAPTER

19

The Cross Talk Between the Central Nervous System, Bone, and Energy Metabolism

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1 INTRODUCTION

Physiology has been approached by two different methodologies. Being the most recent approach, molecular and cellular physiology focuses on studying molecular events occurring within cells, as they relate to a given function or signaling pathway. Earlier on, however, physiology, we will refer to as whole-organism physiology, aimed at identifying interactions between organs through what Claude Bernard called the "milieu intérieur."[1] The study of whole-organism physiology depends upon three fundamental principles. First, no physiological function/process is solely determined by a single organ; this has been verified multiple times through the use of model organisms.[2–4] Second, according to the fundamental concepts of homeostasis forged by W.C. Cannon and L.J. Henderson in the early 20th century, most physiological functions/processes are subjected to a coordinated regulation by several different organs that exert opposite influences on the same function/process.[5,6] This principle is best illustrated by the feedback regulation seen in endocrinology: a regulated organ signals back to a regulating one to limit its influence.[7] Third, regulatory molecules appear during evolution contemporaneously with the functions they regulate not afterward.

In the last half-century, the molecular revolution, especially the advancement of mouse genetics, has greatly transformed our understanding of all biological processes. Specifically, it is the ability to inactivate one gene in a single cell type and in a time-specific manner in the mouse that has led to the identification of many novel endocrine organs and hormones. In this way, mouse genetics has illustrated the value of a whole-organism approach to the study of physiology, provided of course it is conducted one molecule at a time. In this chapter, we review how an approach that is both molecular and whole organism to physiology, has profoundly enriched our views on skeleton biology. To that end, we describe mouse genetics studies pointing toward a coordinated regulation of bone mass and energy metabolism; a central control of bone mass; and the roles of leptin, serotonin, insulin, adiponectin, glucose, and osteocalcin in these pathways.

2 SPECIFIC FEATURES OF BONE AND WHOLE-ORGANISM PHYSIOLOGY

A unique feature of bone is that it is the only tissue in our body that contains a cell type, the osteoclast, whose main function is to resorb (destroy) the tissue in which it resides.[8] This destruction, or resorption, of bone is only one aspect of a biphasic physiological process called bone modeling during childhood, and remodeling during adulthood, in which bone formation invariably follows bone resorption. This constant alternation of bone destruction and bone formation fulfills biological functions of fundamental importance—bone modeling allows the longitudinal growth of the skeleton during childhood and enables one to stand, walk, and run; and bone remodeling that occurs after the arrest of skeletal growth was originally intended to repair micro- and macrodamage, that is, fractures.

Bone is also one of the tissues that cover the largest surface in the body. Hence, the destruction/construction

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activity characterizing bone remodeling that occurs constantly and simultaneously in thousands of locations, requires a large and constant supply of energy to bone cells. The hypothesis that bone (re)modeling is an energetically costly physiological process is supported by several clinical observations. For instance, patients with anorexia nervosa, a disease characterized by very limited food intake, develops growth arrest if children and osteoporosis if adults.[9–19] Clinical experiences tell us another thing: osteoporosis, a low-bone-mass, appears when gonadal function declines.[20,21] Taken these observations together, we hypothesized that bone mass accrual, energy metabolism, and reproduction are all coordinated by endocrine regulation.[22] The possible cross talk between these three distinct physiological systems has several important implications. First of all, as it is bone that justifies the existence of this tripartite regulation, the hormones that make it happen should appear during evolution with bone, not before that. Second, the crucial role of the brain in energy homeostasis suggested that bone (re)modeling may be subject to central regulation. Third, there are likely to be feedback loops that originate from bone and that affect energy metabolism and fertility. Current studies supporting the hypothesis that bone mass, metabolism, and reproduction are linked are presented later.

2.1 Coordinated Control of Bone Mass and Energy Metabolism: Regulation of Bone Mass by Adipocytes

Adipose tissue is not only one of the most important organs of energy storage, but also it can function as endocrine organ by secreting several adipokines that are critical to energy homeostasis. Among them, leptin was from the onset the best candidate to test the hypothesis that bone mass, energy metabolism, and reproduction are linked. Leptin, a 16 kDa hormone secreted by adipocytes,[23] regulates appetite, energy expenditure, and fertility by signaling in the brain.[22,24,25–28] Accordingly, *ob/ob* mice, a natural mutant strain lacking a functional leptin gene, are obese and sterile.[23] The same is true for mice lacking the leptin receptor (*db/db* mice).[29] In addition to its role in energy metabolism, leptin is a powerful inhibitor of bone mass accrual. Indeed, mice or humans lacking leptin or its receptor develop a high bone mass phenotype even though they are hypogonadic, a condition that greatly increases bone resorption.[22] This feature suggested that the inhibition of bone mass accrual may be a major function of leptin. This notion has been verified genetically through the use of a partial gain-of-function leptin signaling model, the 1/1 mice, in which the inhibitory phosphorylation Y285 of the leptin receptor was prevented.[30] Remarkably, 1/1 mutant mice display normal appetite, normal energy expenditure, and normal reproductive functions.[30] Yet, they have a low-bone mass phenotype.[31] This observation indicates that leptin's regulation of bone mass requires a lower threshold of leptin signaling than that needed for regulation of energy metabolism and reproduction.

Previous analysis of leptin's mode of action on appetite and reproduction has demonstrated that these functions are centrally mediated[25,26]; therefore it was expected that leptin's regulation of bone would also be mediated by the central nervous system. Accordingly, several sophisticated genetic studies verified the existence of leptin-dependent central control of bone mass. The most convincing argument among them is that a neuron-specific deletion of *Lepr* recapitulates the bone phenotype of ob/ob mice, whereas an osteoblast-specific one does not.[31] There are also known mediators linking–leptin signaling in the brain to the osteoblasts, the ultimate target cell of leptin. The first one is the sympathetic nervous system, which signals through the β2adrenergic receptor (Adrβ2) present in osteoblasts.[27,28] Mice lacking the 2 adrenergic receptor (Adr 2), the only adrenergic receptor expressed at significant levels in osteoblasts, demonstrate the same high bone mass/high bone formation phenotype seen in *ob/ob* mice, and this phenotype cannot be rescued by ICV infusion of leptin.[27] In the osteoblast, the sympathetic tone recruits several transcriptional components of the molecular clock, cMyc and cAMP response element binding (CREB) protein, to inhibit cell proliferation by decreasing the expression of both D-type Cyclins and AP-1 genes.[32] Sympathetic tone also increases protein kinase A phosphorylation of ATF4, a member of the CREB family of transcription factor that is essential for osteoblast differentiation and function. ATF4 induces expression in osteoblasts of the osteoclast differentiation factor RankL[27,33].[34] Thus, sympathetic tone inhibits bone formation and favors bone resorption, which in turn reduces bone mass accrual. As a result, β-blockers antagonizing Adrβ2 can rescue osteoporosis in mice, rats, and even in humans[28]. The second mediator of leptin regulation of bone mass accrual is the cocaine amphetamine-regulated transcript (CART), a 116-amino acid long peptide that is found in the brain and the general circulation and whose production in the hypothalamus is positively regulated by leptin.[35,36] While mice lacking CART do not present any abnormalities in either body weight or reproduction when fed a normal diet, they demonstrate a late onset low bone mass phenotype caused by a sharp increase in bone resorption.[27,37] Indeed, CART acts on osteoblasts to inhibit *RankL* expression and thereby bone resorption.[27,38] The low bone mass/high resorption phenotype observed

in the absence of CART is similar to the one observed in mice deficient in MC4R, another known target of leptin in the hypothalamus,[39] and CART was identified as the downstream mediator of MC4R in its control of bone resorption.[39] The third mediator of leptin known to act on bone is Neuromedin U, an anorexigenic neuropeptide that is produced by neurons in the brain, as well as nerve cells of the small intestine, and whose expression is regulated by leptin.[40] Neuromedin U-deficient mice show a high bone mass/high bone formation phenotype similar to the one seen in *ob/ob* mice, this phenotype cannot be rescued by ICV infusion of leptin.[41] Additional analyses demonstrated that Neuromedin U mediates the leptin-dependent negative regulation of bone formation by acting on the sympathetic tone to alter osteoblast expression of molecular clock genes.[41] The broader implication of these collective results is that the brain controls bone mass accrual.

2.2 Coordinated Regulation of Bone Mass and Energy Metabolism: The Central Mode of Action of Leptin

To decipher the mechanisms whereby leptin regulates bone mass accrual, one needs to know first where it signals in the brain to fulfill this function. Initially, it seemed that the hypothalamus was where all the action was taking place as the leptin receptor is expressed at high levels in neurons of the arcuate (ARC) and of the ventromedial hypothalamic (VMH) nuclei. Moreover, chemical lesioning of these neurons led to hyperphagia and a massive increase in bone mass and bone formation parameters similar to the one observed in *ob/ob* mice and leptin infusions in the third ventricle of ob/ob mice decreased bone mass and appetite only if these hypothalamic neurons were intact.[28] However, this view was challenged by landmark studies showing that the selective inactivation of Lepr in VMH or arcuate neurons did not affect appetite or bone mass.[42,43] One possible interpretation of these seemingly contradictory results is that leptin requires the integrity of hypothalamic neurons to regulate appetite and bone mass, but does not need to bind with them. In other words, leptin could bind to its receptor in other brain structures than the ARC and VMH nuclei where it would control the synthesis of neurotransmitters that will then act in the hypothalamus. The fact that patients chronically treated with serotonin reuptake inhibitors (SSRIs), a class of drugs preventing serotonin reuptake in neurons, can develop bone loss, hyperphagia, and body weight gain suggested that serotonin and leptin signaling may intersect in the brain. This hypothesis was verified experimentally.[44] Serotonin is a neuromediator made only in neurons of the raphe nuclei and is also a hormone inhibiting bone mass accrual when synthesized by the enterochromaffin cells of the duodenum.[44] However, serotonin does not cross the blood–brain barrier, and thus each pool of serotonin must have different functions.[44] Embryonic or postnatal inactivation of tryptophan hydroxylase 2 (Tph2), the rate-limiting enzyme of serotonin synthesis in the brain, showed that brain serotonin is a powerful activator of bone mass accrual, an activator of appetite, and a regulator of energy expenditure. Because serotonin does not cross the blood–brain barrier, this experiment identified it as the first neuromediator to truly affect the bone mass.[44] Axon tracing and cell-specific and time-specific gene inactivation showed that, by signaling in neurons of the VMH nucleus, serotonin causes a decrease in sympathetic output thereby blocking its dual regulation of bone formation and bone resorption. On the other hand, by acting on ARC neurons, serotonin controls appetite and energy expenditure by favoring expression in these neurons of proopiomelanocortin-α (*Pomc*), melanocortin receptor 4 (*MC4R*), and other genes regulating appetite.[44] In-depth molecular studies showed that in both hypothalamic nuclei serotonin fulfills its function through the transcription factor CREB.

Leptin, instead of acting directly on hypothalamic neurons, signals to its receptor expressed on serotonergic neurons present in the brainstem and this binding causes an inhibition of *Tph2* expression and thereby a decrease in serotonin synthesis.[44] Accordingly, serotonin content in *ob/ob* hypothalamic is abnormally high. Removing one allele of *Tph2* from ob/ob mice normalized their brain serotonin content, appetite, energy expenditure, body weight, and bone mass.[44] Subsequently, the molecular bases of this leptin-serotonin-hypothalamus axis were also deciphered. They include calmodulin signaling in VMH neurons activating the transcription factor cyclic AMP-response-element-binding protein (CREB), which induces the expression of genes involved in catecholamine synthesis. This is important because the mediator acting as a bridge between leptin signaling in the brain and in bone cells is the sympathetic nervous system acting on osteoblasts through the β2 adrenergic receptor (Adrb2) and because β-blockers can limit the risk of osteoporotic fractures.[27,28] The fact that leptin inhibits both bone mass accrual and appetite is consistent with the hypothesis that bone acquisition, an energy-expensive process, must be linked to energy (food) intake, otherwise the risk of organ failure anywhere else in the body, at the time of a growth spurt for instance, would be high. This broader view on leptin suggests a simpler explanation of why this hormone appeared during evolution with bone.

3 EXPANDING BONE BIOLOGY WITHOUT PREMEDITATION

These aforementioned studies together with the powerful influence of food/energy intake on bone (re) modeling revealed by clinical observations raise one interesting question. If energy intake is so important for bone, does bone in turn regulate energy metabolism, in other words is bone an endocrine organ regulating energy metabolism? Genetic studies aiming to address this question resulted in identification of a bone–derived hormone and several novel endocrine functions of bone.

Osteocalcin is an osteoblast-specific protein that is secreted in large amounts in the bone ECM, and the genes encoding osteocalcin start to be expressed around the time bone mineralization begins. Even more suggestive was the fact that through the gamma carboxylation of three glutamic acid residues ("Gla" residues) osteocalcin acquires a high affinity for mineral ions.[45] Those were not only the important features of osteocalcin, they were simply the ones that had attracted the attention of most investigators in the field of bone biology ever since osteocalcin was discovered. And yet, neither the inactivation of *Osteocalcin* nor its overexpression in osteoblasts or other cell types had any effects on ECM mineralization in bones or other tissues.[46,47] However, *Osteocalcin*-deficient (*Osteocalcin*$^{-/-}$) mice had two obvious, albeit unexpected, phenotypes. The most overt phenotype was the dramatic increase in abdominal fat compared to wild-type (WT) littermates. A second more subtle observation was that *Osteocalcin*$^{-/-}$ mice started to breed later and became infertile sooner than WT littermates. Thus, the inactivation of an osteoblast-specific protein secreted in the bone ECM influenced two physiological processes that do not directly involve bones: fat accumulation and reproduction.

The surprising nature of these observations brought back to light other features of osteocalcin that had been overlooked. First, like most peptide hormones, osteocalcin is produced as a pre-/promolecule that is sequentially cleaved in osteoblasts, so that only the mature protein is secreted. Second, like many hormones, osteocalcin is present in the general circulation in the ng/mL range in all species tested and its circulating levels follow a circadian rhythm in humans.[48,49] Thus, the phenotypes of *Osteocalcin*$^{-/-}$ mice, and some biochemical characteristics of osteocalcin, suggested that it might be a peptide hormone secreted by osteoblasts. In fact, osteocalcin is not the only hormone synthesized by bone cells. FGF23 is also synthesized by osteoblasts, reaches the general circulation and acts in the kidney to favor phosphate elimination.[50] There is, however, an important difference between FGF23 and osteocalcin: FGF23 regulates phosphate metabolism, a process intimately linked to bone health itself.[47] In contrast, at least one of the functions of osteocalcin—the regulation of fat mass—had no known link to bone health. This set of observations also raised questions of greater significance: why would bone have any other function besides making bone and if it does, what might these functions be?

4 THE ANTICIPATED FUNCTIONS OF OSTEOCALCIN

The difficulty in testing whether osteoblasts affect energy metabolism through osteocalcin is that energy metabolism includes a loosely defined aggregate of processes ranging from food intake to the utilization of nutrients in peripheral tissues. The approach used to circumvent this difficulty was to coculture osteoblasts with other cell types or organ explants that secrete hormones regulating key aspects of energy metabolism. The two structures tested in this experimental scheme were pancreatic islets, given the number of aspects of energy metabolism regulated by insulin, and adipocytes, as leptin indirectly regulates osteoblast function.[22,27,28,44] Supernatants of WT osteoblasts enhanced the expression of *Insulin* in pancreatic islets and of *Adiponectin* in adipocytes. Several criteria of specificity strengthened these findings. For instance, the supernatant of fibroblasts, the cell type that is the most closely related to osteoblasts, failed to stimulate *Insulin* or *Adiponectin* expression. A second criterion of specificity is that among all the hormones expressed by pancreatic islets and adipocytes, only *Insulin* and *Adiponectin* expression was affected by the supernatant of osteoblast cultures. However, none of these changes in gene expression were observed when using the supernatants of *Osteocalcin*$^{-/-}$ osteoblasts identified osteocalcin as an osteoblast-derived hormone that affects *Insulin* and *Adiponectin* expression. These studies also identified the active form of osteocalcin since addition of uncarboxylated but not carboxylated osteocalcin increased Insulin and *Adiponectin* expression in islets and adipocytes, respectively.[51]

The in vivo analysis of the endocrine functions of osteocalcin has greatly benefited from the existence of mouse models of global and osteoblast-specific loss- (*Osteocalcin*$^{-/-}$ and *Osteocalcin*$_{osb}{}^{-/-}$ mice) and gain-of-function of osteocalcin (*Esp*$^{-/-}$ and *Esp*$_{osb}{}^{-/-}$ mice), each model serving as a mirror image control for the other. Consistent with the results of cell-based assays, insulin secretion and β-cell proliferation/mass were both decreased in *Osteocalcin*$^{-/-}$ mice. These mutant mice were also glucose-intolerant and insulin-resistant when fed normal chow, whereas the opposite was true in *Esp*$_{osb}{}^{-/-}$ mice. It remains to be determined what appears as an insulin resistance is rather indicative of the fact that osteocalcin may favor glucose uptake, independently of insulin. Explaining in part their changes in fat mass, energy expenditure was significantly decreased in *Osteocalcin*$^{-/-}$ and

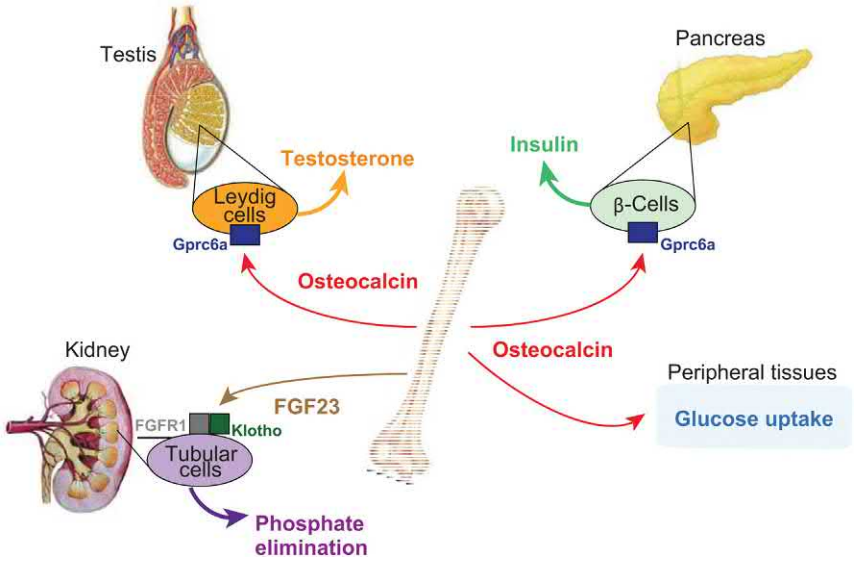

FIGURE 19.1 Schematic representation of the main organs whose functions are affected by osteocalcin (pancreas and testes) and FGF23 (kidney), the two osteoblast-derived hormones. Gprc6a is the receptor for osteocalcin in pancreatic β-cells and Leydig cells of the testes; FGFR1 and Klotho mediates FGF23 signal in tubular cells of the kidney.

increased in $Esp_{osb}^{-/-}$ mice.[51] These observations were recently extended to human pancreatic islets, in which the addition of uncarboxylated osteocalcin increased the expression of the *Insulin* and *Cyclind2* and *Cdk4* genes, two genes needed for β-cell proliferation.[52] Moreover, circulating osteocalcin levels are inversely correlated in adults with fasting glucose, circulating insulin levels, BMI, and body fat.[53]

These results demonstrate that osteocalcin is a bone-derived hormone that regulates β-cell proliferation, *Insulin* expression, and insulin secretion in mice and most likely in humans (Fig. 19.1). They also suggested that the hormonally active form of osteocalcin is the decarboxylated one. This was subsequently confirmed through genetic means.[54] The same coculture assay was used to test whether osteoblasts and presumably osteocalcin favor reproductive functions. Surprisingly, given that osteoporosis is primarily a disease of postmenopausal women, neither the supernatant of osteoblasts nor the uncarboxylated osteocalcin influenced the secretion of estrogen by ovary or follicular cells. In contrast, recombinant osteocalcin and supernatants of WT, but not of $Osteocalcin^{-/-}$ osteoblast cultures induced the secretion of testosterone by Leydig cells of the testes. Here again, the experiment was controlled in several ways; supernatants of no other mesenchymal cell types could increase testosterone secretion by Leydig cells of the testes and osteocalcin did not increase the secretion of estradiol, a derivative of testosterone, by Leydig cells. Extending these cell-based assays, the analysis of cell-specific loss- and gain-of-function mouse models of osteocalcin function, as well as the treatment of Leydig cells with osteocalcin, showed that uncarboxylated osteocalcin signals directly to Leydig cells to favor the expression of all genes encoding the enzymes necessary for testosterone synthesis, but does not affect the expression of *Cyp19* that is needed for the aromatization of testosterone into estradiol. As a result, male mice lacking osteocalcin show typical features of hypotestosteronemia, for example, low sperm count and lower weight of the epididymis and testes (Fig. 19.1). In contrast, the circulating levels of luteinizing hormone (LH) that favor testosterone secretion were increased in $Osteocalcin^{-/-}$ mice.[55]

4.1 The Other Side of Osteocalcin

Once the predicted endocrine functions of osteocalcin were demonstrated, a systematic search for other target organs for this hormone began. With regard to this, $Osteocalcin^{-/-}$ mice, regardless of gender, are markedly more docile than their WT littermates. Initially, this docility was ascribed to the low circulating level of sex steroid hormones that was suspected to exist in $Osteocalcin^{-/-}$ mice of both sexes. However, once it was shown that osteocalcin regulates sex steroid hormones synthesis only in male mice, this phenotype took another dimension and was analyzed rigorously. The docility of female $Osteocalcin^{-/-}$ mice could be traced to a decrease in the synthesis of all monoamine neurotransmitters and to an increase in the synthesis of GABA. In agreement with the extent of these abnormalities and the biological importance of these neurotransmitters, $Osteocalcin^{-/-}$ mice displayed severe behavioral phenotypes, such as increased anxiety and a profound deficit in spatial learning and memory. These abnormalities were caused by a lack of signaling of osteocalcin in the brain since delivery of the hormone in the brain through intracerebroventricular

infusion corrected them. Osteocalcin affects neurotransmitter synthesis because it crosses the blood–brain barrier and binds specifically to serotonergic neurons of the raphe nuclei in the brainstem, to neurons of the CA3 region of the hippocampus and of the dopaminergic nucleus of ventral tegmental area in the midbrain. Hence, these experiments demonstrated a significant influence of bone on neurotransmitter synthesis in the brain and on cognition[55a].

As unexpected as these findings were, another observation was even more surprising. A histological analysis showed that the hippocampi of *Osteocalcin*$^{-/-}$ mice were hypoplastic compared to those of WT littermates. This developmental defect was puzzling because *Osteocalcin* is not expressed during mouse embryogenesis before the last 2 days of gestation). Studying this observation revealed that maternally derived osteocalcin crosses the placenta and favors hippocampus development by preventing neuronal apoptosis. As a result *Osteocalcin*$^{-/-}$ mice exhibited a more severe cognitive deficit when their mothers were also *Osteocalcin*$^{-/-}$. Conversely, providing osteocalcin once a day to pregnant *Osteocalcin*$^{-/-}$ mothers normalize the development of the hippocampus and partly rescue the deficit in memory in their *Osteocalcin*$^{-/-}$ progeny. The observations that maternal osteocalcin contributes to the development of the brain in the embryo before it can make it, identifies osteocalcin, as a molecule responsible for the beneficial influence of the mother's health on the development of the brain and cognitive functions of the offspring (Fig. 19.2). The notion that maternal osteocalcin is present in the embryo before expression of *Osteocalcin* also suggests that osteocalcin may have a broader than anticipated influence on the health of the offspring.

5 QUESTIONS RAISED BY THE FUNCTIONS OF OSTEOCALCIN

The nature, extent, and number of the endocrine functions of osteocalcin raised interesting new questions. Key among them is the identity of the receptor for osteocalcin and the biological relevance of these findings beyond genetically modified mice, in other words whether the endocrine functions of osteocalcin observed in mice are conserved in humans. These questions were addressed through the study of the reproductive functions of osteocalcin. Osteocalcin's stimulation of testosterone secretion by Leydig cells follows a bell-shaped curve reminiscent of what is observed when a ligand binds to a G protein coupled receptor (GPCR). Further supporting this notion, treating Leydig cells with osteocalcin-induced production of cAMP while it failed to induce tyrosine phosphorylation, ERK activation, or intracellular calcium accumulation. These data justified a search for a GPCR that would be expressed in Leydig cells of the testes but not in follicular cells of the ovary and would transduce the osteocalcin signal. This screen identified a single candidate, Gprc6a.[55] Of note, *Gprc6a*$^{-/-}$ mice display metabolic and male reproductive abnormalities similar to those seen in *Osteocalcin*$^{-/-}$ mice.[56] Analysis of cell-specific gene deletion and compound mutant mice lacking one allele of *Osteocalcin* and one allele of *Gprc6a* in Leydig cells or β-cells only verified that Gprc6a mediates the osteocalcin signal in these cell types.[55]

Remarkably, *Gprc6a* is not expressed in any of the brain structures to which osteocalcin binds, *Gprc6a*$^{-/-}$ mice have normal neurotransmitter accumulation in the brain, normal hippocampal development, and normal

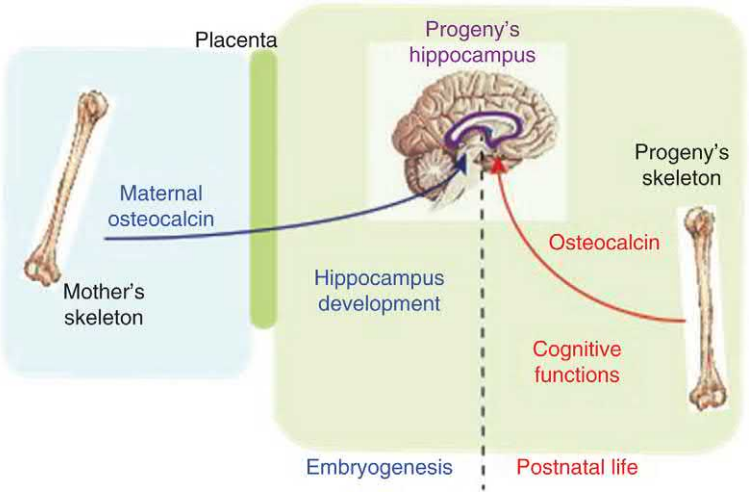

FIGURE 19.2 **Schematic representation of the functions of osteocalcin in the brain.** Maternally derived osteocalcin crosses the placenta, reaches the developing brain, and favors hippocampal development. In adult animals osteocalcin crosses the blood brain barrier regulates the synthesis of various neurotransmitters, prevents anxiety, and favors spatial learning and memory. The receptor for osteocalcin in the brain has not been identified yet.

cognitive functions. These findings ruled out that the cognitive defects seen in *Osteocalcin*$^{-/-}$ mice are secondary to their metabolic or endocrine abnormalities, as these abnormalities are equally severe in *Osteocalcin*−/− and *Gprc6a*$^{-/-}$ mice; on the other hand, they raise the question of the identity of the receptor(s) of osteocalcin in the brain.

Providing evidence that the endocrine functions of osteocalcin also exist in humans was helped by the fact that the reproductive phenotype of *Osteocalcin*$^{-/-}$ mice, low testosterone levels and high-LH circulating levels, bears resemblance to peripheral testicular insufficiency in humans. Sequencing all exons of *Osteocalcin* and *Gprc6a* in 59 of these patients identified 2 unrelated individuals harboring the same dominant negative mutation in a conserved residue of the GPRC6A extracellular domain, a mutation that could not be found in control individuals. Of note, glucose tolerance was also abnormal in both patients. Although more studies are needed, these data suggest that signaling through GPRC6A, presumably by osteocalcin, is required for testosterone synthesis by Leydig cells in humans.

Other questions raised by this work were to determine the potential influence on osteoblasts on insulin and adiponectin, the two hormones whose expression is regulated by osteocalcin. Addressing the relationship between bone and pancreas from the viewpoint of insulin helped explain how osteocalcin, a constituent of the bone ECM, becomes a hormone. Through its signaling in osteoblasts, insulin inhibits the expression of *Osteoprotegerin*, an inhibitor of osteoclast differentiation, thus insulin signaling in osteoblasts favors bone resorption. As the only known means to decarboxylate a protein once outside the cell is a low pH, such as the one reigning within the resorption lacuna, insulin signaling in osteoblasts allows the carboxylated form of osteocalcin present in the bone ECM to be decarboxylated on one particular Gla residue and released in the general circulation. These results that identified bone as a more significant insulin target organ than previously thought led to the demonstration that bone is a site of insulin resistance in diabetic mice. The demonstration that osteocalcin also regulates adiponectin expression led to the realization that in unchallenged mice adiponectin is a powerful regulator of bone formation (Fig. 19.3).[57]

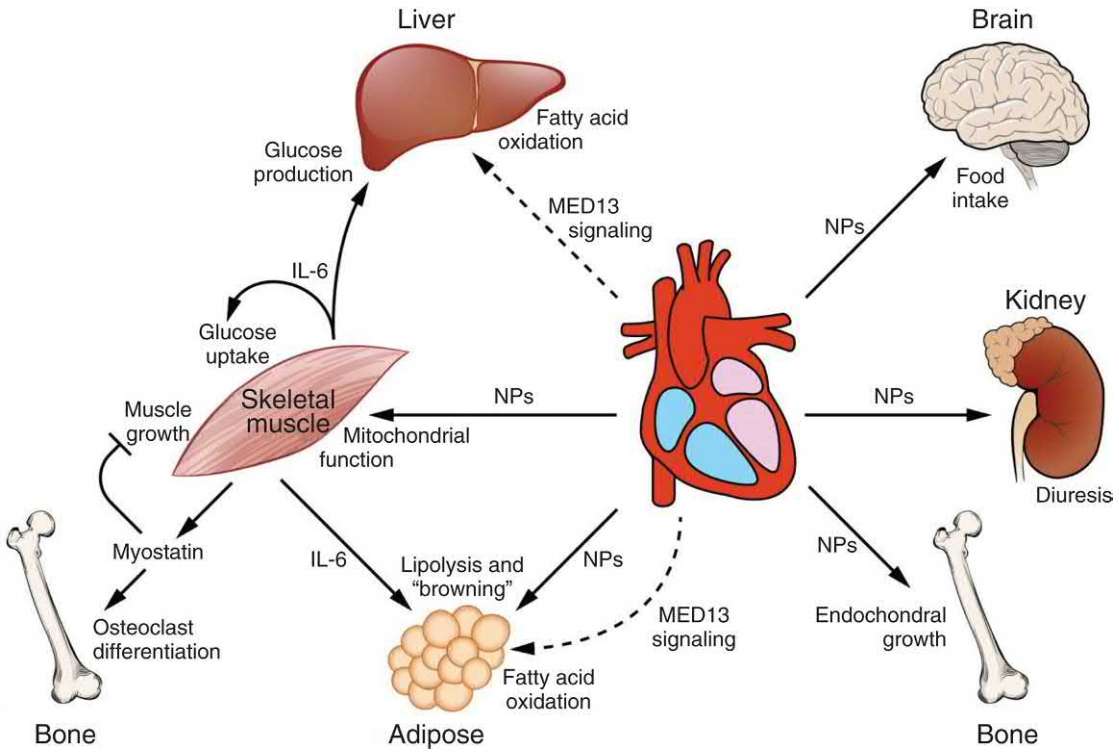

FIGURE 19.3 Schematic representation of endocrine signaling from striated muscles to distal tissues. Myostatin and IL-6 signal from skeletal muscle to distal tissues and also exert autocrine functions to suppress muscle growth and enhance glucose uptake, respectively. Natriuretic peptides (NPs) released from the heart regulate diverse processes in distal tissues. The mediator subunit MED13 acts in the heart to enhance fatty acid oxidation in liver and white adipose tissue. The factor(s) that mediate(s) signaling from cardiac MED13 to distal tissues have not been identified.

More topical questions remain to be addressed. For instance, we do not know how osteocalcin favors energy expenditure or glucose uptake in peripheral tissues. Likewise, the influence of maternal osteocalcin on glucose homeostasis in the offspring has not been accounted for. The fact that another hormone besides LH, be it osteocalcin made in bone, favors sex steroid synthesis in the male but not female gonads raises the question of whether such a hormone exists in females and, if so, which tissue synthesizes it. As for the cognitive functions of osteocalcin, not only do we need to identify its receptor in the brain but we also need to determine whether osteocalcin can improve cognition in WT animals, both young and old. Given the progressive aging of the general population and the paucity of drugs to treat or prevent the age-related decline in cognition this is a question of critical importance. There is one important aspect of bone biology that is worth mentioning in that context. Bone mass remains relatively constant for the first 3 decades of life and begins to decrease thereafter. This decrease is greatly accelerated at the time of menopause in women but eventually aging is associated with a marked decrease in bone mass in both sexes. This observation raises the question of whether bone in its endocrine capacity may in fact delay the age-related decrease in some physiological functions and may prevent the appearance of some aging manifestations. We now have the tools to address the test hypothesis in vivo.

But again the question of broader significance looming behind, the apparently disparate nature of the physiological functions regulated by osteocalcin, is to understand, what were the evolutionary advantages of the endocrine function of bone? Addressing this question in full will require identifying all functions of osteocalcin and possibly the ones of other hormones made by bone cells. Yet, one could use features shared by the known functions of osteocalcin to try to get at the logic of bone as an endocrine organ. It is obvious that male fertility is needed for the survival of any species. To a certain extent, the same can be said of the ability to utilize glucose in peripheral tissues and to remember where food sources and predators are located, for animals living in a hostile environment, such as the ones in which bony vertebrates lived early on. Under this light, the endocrine functions of osteocalcin and the classical functions of bone suggest that this tissue may have conferred evolutionary advantages of two types. By allowing walking and running, bone enabled animals to escape danger and to find food. At the same time, through its endocrine functions bone may have provided a means of survival in hostile environments. This view on the endocrine functions of bone is mainly a tool to search for additional physiological processes it may regulate. One may expect that other bone–derived hormones will be characterized in the future.

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CHAPTER

20

Fetal Control of Calcium and Phosphate Homeostasis

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1 INTRODUCTION

Regulation of mineral and bone homeostasis in the adult is largely achieved through the interactions of parathyroid hormone (PTH), 1,25-dihydroxyvitamin D (calcitriol), fibroblast growth factor-23 (FGF23), calcitonin, and the sex steroids. Over the decades our knowledge had slowly developed from studies in humans and animals through surgical and pharmacological approaches that disrupt the normal regulation of mineral homeostasis, and studies of naturally occurring diseases characterized by altered mineral homeostasis. Our knowledge has rapidly expanded over the past 2 decades with the development of genetically engineered mouse models that enable the function of a single gene to be deduced by eliminating it or over-expressing it in the intact organism, and of molecular methods that enable detailed in vitro analysis of the interactions between ligand and receptor.

In contrast to the adult, comparatively less is known about the regulation of mineral and bone homeostasis in the fetus. This is largely because studying fetuses poses significant technical challenges. Human data have been limited to the analysis of cord blood samples in newborn infants, and pathological examination of embryos and fetuses that died at or before birth. Consequently, much of our understanding about human regulation of fetal mineral homeostasis must be inferred from studies in animals. Genetically engineered mouse models have greatly facilitated the study of fetal mineral homeostasis, but even then the small size of murine fetuses (2.0–2.5 cm and 0.8–1.1 g at term) makes systematic study of them particularly challenging and slow. Consider that the recoverable blood volume of a term fetal mouse is 50–60 μL of whole blood from which 30–40 μL of serum or plasma may be obtainable. Many assays for minerals and hormones require sample sizes of 20–300 μL. In an adult it is possible to measure multiple hormones and minerals from a single blood sample and to collect samples at multiple time points in the same individual, such as in a longitudinal study or as a time course in response to an acute challenge (injection of EDTA, PTH, etc.). But in studies of fetal mice only one blood sample can be obtained and it is inevitably a terminal experiment. Consequently cross-sectional data are obtained from studies of fetal mice, and multiple blood samples may have to be pooled for one measurement.

As this chapter will show regulation of fetal mineral homeostasis differs from the adult (Fig. 20.1). Skeletal patterning begins in the embryo but it is during fetal development that bone formation and mineralization accelerate. The placenta actively transports calcium, phosphorus, and magnesium in order to mineralize the skeleton before birth and maintain appropriate extracellular concentrations of these minerals for normal cellular functioning. The intestines play an insignificant role in fetal mineral metabolism, but after the placental pump is lost at birth, intestinal absorption of minerals becomes critical.

Each section of this chapter will review animal data and then specify whether any human data are available and how they agree or not with the animal data. To avoid exceeding specified limits on the reference list, two recent comprehensive reviews will be cited in place of many original research papers.[1,2]

2 OVERVIEW OF FETAL AND NEONATAL MINERAL METABOLISM

2.1 Animal Data

In all mammalian fetuses that have been studied (rhesus monkeys, sheep, cattle, rats, mice, pigs, and horses) the

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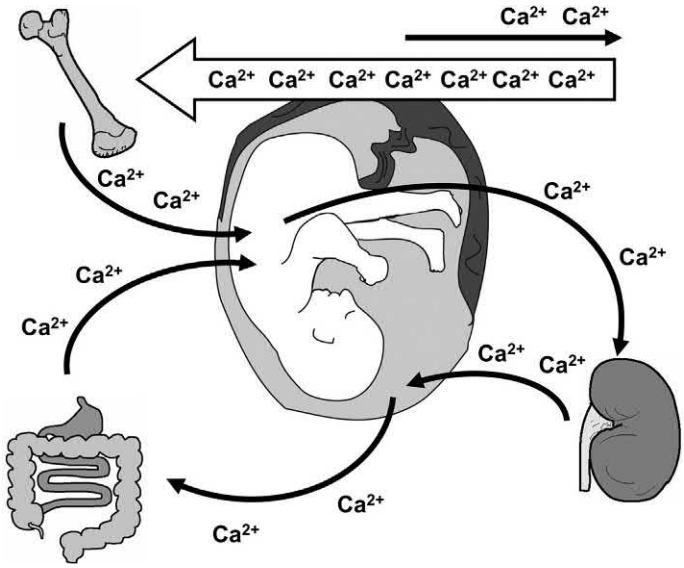

FIGURE 20.1 Circulation of mineral within the fetal–placental unit. Calcium is represented here but these statements apply to phosphorus (phosphate) and magnesium as well. At the top right, the main flux of mineral is across the placenta and through the fetal circulation into bone; however, some mineral returns to the maternal circulation (backflux). On the bottom right, the fetal kidneys filter the blood and excrete mineral into urine, which in turn makes up much of the volume of amniotic fluid. On the bottom left, amniotic fluid is swallowed and its mineral content can be absorbed by the fetal intestines, thereby restoring it to the circulation. The renal–amniotic–intestinal loop is likely a minor component for fetal mineral homeostasis. On the top left, although the net flux of mineral is into bone, some mineral is resorbed from the developing skeleton to reenter the fetal circulation. If placental delivery of mineral is deficient, fetal secondary hyperparathyroidism ensues, which causes more substantial resorption of mineral from the fetal skeleton, reduced skeletal mineral content, and possible fractures occurring in utero or during the birthing process.

serum calcium, ionized calcium, and phosphorus are higher than simultaneous maternal values.[1] This means the fetus is exposed to a high calcium × phosphorus product that can cause soft tissue calcifications in the adult but seems nonhazardous in utero. Serum magnesium is more variable with some studies showing slight increases, decreases, or no change as compared to the maternal value.

The high fetal blood calcium exceeds what the calcium sensing receptor (CaR) sets in the adult, and this level is maintained independent of the maternal blood calcium. Fetal rats remain normocalcemic despite maternal hypocalcemia caused by a calcium-restricted diet, severe vitamin D deficiency, or thyroparathyroidectomy.[1] Similarly fetal blood calcium in mice is unaffected by chronic maternal hypercalcemia (ablation of CaR) or hypocalcemia [ablation of vitamin D receptor, 1α-hydroxylase (CYP27b1), or PTH].[3–6] Chronic maternal hypophosphatemia also has no effect on fetal serum calcium or phosphorus.[7] The maternal blood calcium has also been acutely altered in rodents and primates by infusing calcium, calcitriol, calcitonin, PTH, or EDTA, and these studies have generally shown minimal or no effect on the fetal blood calcium.[1] However, when chronic maternal hypocalcemia in rats was induced at midgestation by parathyroidectomy, the fetuses were normocalcemic for several days but became hypocalcemic during the last few days of gestation.[1] The fetal skeleton rapidly accretes mineral during late gestation, and so it appears that the fetus may be unable to maintain a high fetal serum calcium during that time if the supply in the maternal circulation is very low.

As relative fetal hypercalcemia is robustly maintained across all species studied and often despite maternal hypocalcemia or hypercalcemia, it must have physiological importance. However, survival to the end of gestation is unaffected by significant hypocalcemia in *Pthrp* null, *Pthr1* null, *Hoxa3* null, *Trpv6* null, *Pth* null, *Gcm2* null, *Gp130* null, and *Hoxa3/Pthrp* double mutant fetuses.[1,5,8–13] On the other hand, it is possible that survival after birth is aided by fetal hypercalcemia. The onset of breathing is associated with a marked decrease in the blood calcium by 40% in rodents within the first 12 h after birth, followed by an increase to the adult value during the succeeding day.[1] A lower blood calcium in utero may lead to an even lower trough level in calcium after birth, thereby increasing the risk of tetany and death. Early postnatal mortality of hypocalcemic *Pthrp* null, *Pthr1* null, *Hoxa3* null, *Pth* null, and *Gcm2* null fetuses are conceivably due in part to this mechanism.[1,8,9,14] But the most convincing physiological role for fetal hypercalcemia is to facilitate normal mineralization of the fetal skeleton. Studies in fetal mice have shown that when the serum calcium is at or below the maternal level, then skeletal mineral content at term is significantly reduced.[5,8]

The fetal circulation is characterized by low levels of PTH, calcitriol, and FGF23, and high levels of

PTH-related protein (PTHrP) and calcitonin.[1] PTH appears to be suppressed by the CaR in response to the high fetal blood calcium. Consequently, when CaR is ablated in mice, both serum calcium and PTH rise above the fetal norm.[3] 25-hydroxyvitamin D [25(OH)D] readily crosses the placenta and results in cord blood 25(OH)D levels that are 75%–100% of the maternal value at term.[1] The low-calcitriol levels are due to suppression of the renal CYP27b1 by high serum calcium and phosphorus, low PTH, as well as relatively high activity of the catabolic enzyme, 24-hydroxylase (CYP24A1). The fetal kidneys are capable of upregulating CYP27b1; fetal hyperparathyroidism due to ablation of CaR results in high-calcitriol levels.[3]

As discussed in more detail in later sections, animal studies have shown that absence of parathyroids, PTH, or PTHrP each causes fetal hypocalcemia and hyperphosphatemia,[1,5,8,9,14] whereas fetal absence of calcitonin,[15] vitamin D,[16–18] calcitriol,[6,19] vitamin D receptor,[4,20] or FGF23,[7] do not disturb fetal blood calcium or phosphorus.

Minerals enter the fetus predominantly via the placenta (Fig. 20.1); consequently, control of active mineral transfer across the placenta explains many of the differences between mineral homeostasis of the fetus and the adult. As noted in more detail later, PTHrP regulates placental calcium and possibly magnesium transfer while PTH may also play a role[5,14,21–23]; the regulators of active placental phosphorus transport remain unknown.[1] During the interval of active calcium transfer, the placenta markedly upregulates expression of genes involved in calcium transport, and the fetal skeleton accretes mineral rapidly such that rats attain 95% of skeletal calcium during the last 5 days of gestation.[4,24–27] A small amount of mineral is excreted by the kidneys into the amniotic fluid, which confirms that mineral is not permanently lost since the amniotic fluid can be swallowed and the mineral content will then be reabsorbed. However, this route of mineral entry likely plays an insignificant role in fetal mineral homeostasis.

At birth the mechanisms controlling mineral homeostasis rapidly change. Placental infusions of minerals and hormones are lost upon cutting the umbilical cord, and breathing causes a rise in blood pH. These factors precipitate a 40% decline in blood calcium of rodents after birth. Phosphorus increases over the same interval and then declines. PTH rises to adult normal values by 24–48 h after birth and this precedes (and likely causes) a rise in calcitriol.[1]

In contrast to the fetus, the neonate is dependent upon the intestines for supply of minerals and for the kidneys to reclaim much of the renal filtered load of calcium, and to excrete excess phosphorus. Initially calcium absorption in neonatal rodents is passive and nonsaturable.[1] The high-lactose content of milk increases paracellular diffusion of calcium in the distal small bowel and net bioavailability of dietary calcium.[28] As the neonate matures, passive absorption of calcium declines, enterocytes upregulate expression of the vitamin D receptor and associated calcium transporting genes and proteins, and calcium absorption becomes an active, saturable, and calcitriol-dependent process.[1]

2.2 Human Data

Available data from developing humans are consistent with the animal data. Serum calcium, ionized calcium, magnesium, and phosphorus are all higher than maternal values.[1] The high serum calcium has been demonstrated as early as 15–20 weeks of gestation by fetoscopy. This fetal hypercalcemia appears to be maintained even in situations of maternal hypocalcemia due to severe vitamin D deficiency or hypoparathyroidism.[1] Cord blood measurements in normal fetuses have shown low levels of PTH, calcitriol, and FGF23, and high levels of PTHrP and calcitonin.[1,29] About 80% of the mineral content present in a term fetus is accreted during the third trimester,[30] during which active placental calcium and phosphorus transport takes place.

The onset of breathing causes a 20%–30% fall in the blood calcium followed by an increase to adult levels over the succeeding 24 h, and increases in PTH followed by calcitriol.[31] Intestinal calcium absorption in newborns (more so in preterm babies) is largely passive and nonsaturable,[32,33] facilitated by the high lactose content of human milk.[34,35] As the neonate matures, the intestine become less able to passively absorb calcium and instead active, saturable, and calcitriol-dependent intestinal calcium absorption becomes dominant.[32,36,37] This developmentally programmed maturation of the neonatal intestine explains why preterm babies do not respond to calcitriol but are dependent upon passive absorption of mineral until they become more mature.

3 OVERVIEW OF PLACENTAL MINERAL TRANSPORT

3.1 Animal Data

Active placental transport of calcium and other minerals is required because diffusional flow is insufficient to account for the required rapidity of transfer, and these mineral exchanges occur against electrochemical gradients.[1] Similar to intestinal calcium absorption, calcium presumably enters via gated calcium channels [such as transient receptor potential vanilloid 6 (TRPV6)] in the maternal-facing basement membranes, binds to intracellular proteins in order to shuttle to the opposite basement membrane, and then is pumped out at the fetal-facing

basement membranes by Ca^{2+}-ATPase. In rodent placentas, calcium is likely transported across trophoblasts and cells of the intraplacental yolk sac.

Calbindin-D9k is likely one of the binding proteins that shuttle calcium within trophoblasts and intraplacental yolk sac cells. Its placental expression begins at midgestation but increases many-fold during the interval of rapid placental calcium transfer in rodents. At the same time expression of Ca^{2+}-ATPase doubles while TRPV6 increases 14-fold.[1] All three calciotropic genes colocalize within trophoblasts but in rodent placentas the most intense expression is within the intraplacental yolk sac, a structure that also expresses other calciotropic genes at the highest intensity compared to the surrounding trophoblasts.[10,38] The significance of this yolk sac activity is discussed later.

Whether calbindin-D9k, Ca^{2+}-ATPase, and TRPV6 are essential for placental calcium transport is unclear. Placental calcium transfer has not been assayed in the available knockouts of calbindin-D9k (*S100g*) and Ca^{2+}-ATPase (*Pmca1*, *Pmca2*, and *Pmca4*).[1] Calbindin-D9k expression is reduced within the intraplacental yolk sac of two mouse models that have subnormal rates of placental calcium transfer (*Pthrp* null and *Trpv6* null),[10,38] but whether that is a cause or consequence of reduced placental calcium transfer is unknown. Knockout of *Trpv6* does not reduce intestinal calcium absorption in adult mice, but placental calcium transfer is reduced 40%, skeletal ash weight at term is 50% of wild-type (wt) values, and *Trpv6* null fetuses are severely hypocalcemic.[10] Therefore, TRPV6 must play an important role in placental calcium transfer and fetal calcium homeostasis. Since *Trpv6* null fetuses have reduced placental calcium transport while the adults have no impairment of intestinal absorption, it remains conceivable that *S100g*, *Pmca1*, *Pmca2*, or *Pmca4* null mice might have impaired placental calcium transport despite normal intestinal calcium absorption as adults.

There are significant structural and functional differences among placentas of the various species that have been studied.[1] Placentas of sheep, goats, and pigs have a cotyledonary structure of 60–70 individual units (cotyledons) that are spread over the entire uterine wall. The microscopic structure is epitheliochorial, which means that maternal and fetal circulations are separated by full thicknesses of maternal and fetal tissues. Calbindin-D9k expression is most concentrated in the interplacentomal region that has been postulated to be an important site of calcium transfer between mother and fetus in ruminants. The placentome and interplacentomal region also contain the highest expression of Ca^{2+}-ATPase.

Placentas of primates and rodents are discoid, in which placental tissue is confined to a single plate. The microscopic structure is hemochorial, meaning that fetal trophoblasts have fully invaded the uterine tissue such that maternal blood comes in direct contact with the fetal chorion. Consequently the barriers to exchange of nutrients and waste are reduced as compared to epitheliochorial placentas. Rodent placentas are hemo*trichorial* with three layers of trophoblasts interposed between the maternal and fetal circulations. Trophoblasts form a labyrinthine series of small channels bathed in maternal blood. Rodent placentas also contain the intraplacental yolk sac, a bilayered membrane interposed between fetal and maternal blood vessels (Fig. 20.2). A channel which lies

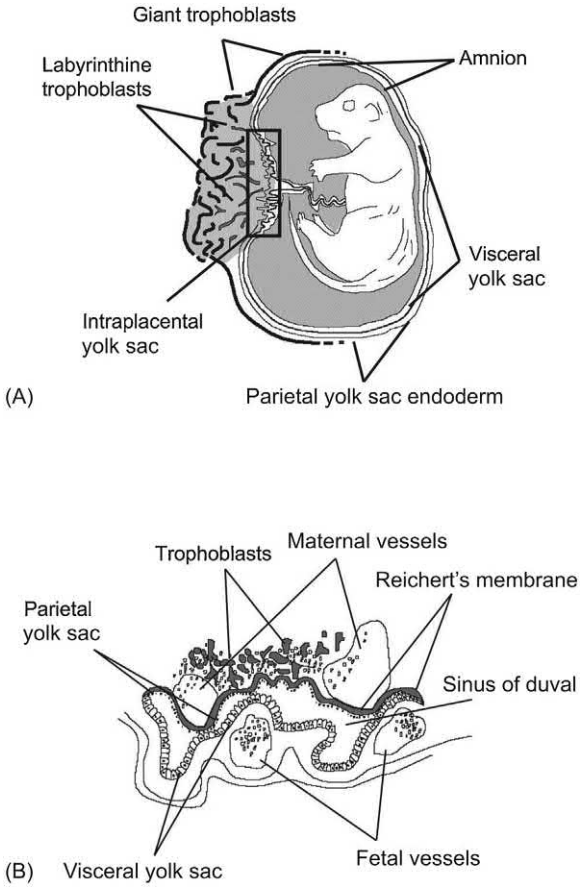

FIGURE 20.2 Anatomy of the intraplacental yolk sac. By the time the mature placenta has formed (A), the compressed yolk sac lines the uterine cavity (decidua) that is not in contact with the placenta, and it overlies the dome of the placenta. The parietal layer and Reichert's membrane are in contact with the decidua and the dome of the placenta, while the columnar or visceral yolk sac layer is in apposition to this layer, separated by the yolk sac cavity. The yolk sac bilayer that overlies the dome of the placenta forms finger-like projections into the placenta near the insertion of the fetal vessels [boxed area in (A), the intraplacental yolk sac]. The intraplacental yolk sac is positioned between maternal and fetal blood spaces, as visualized in detail in (B). Within this structure, the parietal layer and Reichert's membrane overlie maternal blood spaces and vessels, while the columnar layer overlies fetal blood vessels. Between these layers is the sinus of Duval, which also communicates with the yolk sac cavity and the uterine lumen. *Source: Reproduced with permission from Kovacs CS, Chafe LL, Woodland ML, McDonald KR, Fudge NJ, Wookey PJ. Calcitropic gene expression suggests a role for intraplacental yolk sac in maternal–fetal calcium exchange.* Am J Physiol Endocrinol Metab *2002;***282***(3):E721–32, copyright 2002 American Physiological Society.*[38]

in between the two layers is called the sinus of Düval. Although rodent trophoblasts express many calciotropic genes, the expression of calbindin-D9k, Ca^{2+}-ATPase, PTHrP, PTH/PTHrP receptor (PTH1R), TRPV6, vitamin D receptor, and CaR are most intense within the intraplacental yolk sac.[1,38] PTH mRNA has recently been found to be expressed in murine placenta but localization studies have not been done owing to lack of suitable antibodies.[5] Cyp27b1 and Cyp24a1 are expressed by trophoblasts, as are FGF23's coreceptor Klotho, all four FGF receptor subtypes, and all three sodium phosphate cotransporter two subtypes (NaPi2b > NaPi2a and NaPi2c).[7,39] Very-low-level placental expression of FGF23 was confirmed by comparison of normal and *Fgf23* null placentas.[7]

Due to its positioning between the maternal and fetal vessels and its intense expression of calciotropic factors, the intraplacental yolk sac may be a site of maternal–fetal calcium exchange. Moreover the intraplacental yolk sac and its sinus of Düval are continuous with corresponding structures of the extraplacental yolk sac that in turn overlie the uterine epithelium. Hence it is possible that secretions from the uterine epithelium cross the parietal layer of the extraplacental yolk sac to reach the sinus of Düval, travel to the placenta, and enter the fetal circulation across the columnar epithelium within the intraplacental yolk sac. Precedent for this concept is that egg shell calcification proceeds from exuberant uterine secretions of calcium and other minerals. The intraplacental yolk sac may have evolved in rodents as an alternate route for active calcium transport in order to meet the calcium demands created by large litters and a short gestation period. However, owing to its microscopic size and other factors, it hasn't been technically feasible to determine whether calcium or other minerals are being exchanged at this site. A genetic knockout resulting in loss of the intraplacental yolk sac would be one way to test this. Platelet-derived growth factor-alpha is intensely expressed in the intraplacental yolk sac, and a naturally occurring deletion of this gene results in placentas devoid of this structure.[40] However, most pups die at midgestation, too soon for placental calcium transport or skeletal mineralization to be studied.

Two main approaches have been used to assay transfer of calcium, phosphorus, or magnesium across the placenta. The first is an in situ placental perfusion technique used in sheep, goats, and rats. In this procedure the dam remains anesthetized, the fetus is removed, the placenta is connected to a semiclosed circuit, and either autologous fetal blood or a blood substitute is used to perfuse the circuit at a set flow rate and pressure. ^{45}Ca and ^{51}Cr-EDTA (a diffusional marker) are infused into the mother, and repeated sampling from the maternal circulation and umbilical vein enables calculation of radioisotope clearance rates. ^{32}P or ^{28}Mg have also been administered in combination with ^{51}Cr-EDTA. Peptides or pharmacologic agents can be infused into the umbilical artery while the umbilical vein is continuously sampled to determine if the calcium (or phosphorus or magnesium) concentration is altered by the intervention. The main limitations of this technique are that it is done in a single placenta, in the absence of normal fetal regulation, and with the perfusate pressure and rate at arbitrarily set levels.

The in situ placental perfusion technique has recently been miniaturized to the small scale of fetal mice, but in order to successfully catheterize the umbilical vessels the vasculature is first maximally dilated with nitroglycerin.[41,42] ^{45}Ca alone is administered to the mother and only single measurements of ^{45}Ca in the maternal circulation or placental circuit can be obtained. The use of nitroglycerin may confound the experiment by creating artifactual differences between wt and mutant placentas if they differ in factors, such as ambient blood pressure, structure, or responsiveness to vasodilators.

A second technique used in mice keeps the fetus intact and in control of placental function. The pregnant mother is anesthetized for a few seconds in order to receive an intracardiac injection containing ^{45}Ca and ^{51}Cr-EDTA (or ^{32}P and ^{51}Cr-EDTA), and as early as 5 min after the maternal injection, the fetuses are removed so that the amount of isotopes accumulated within each fetus can be measured. As ^{51}Cr-EDTA crosses the placenta by passive diffusion, it serves as a control for differences in flow rate between the individual placentas in one litter. The relative rate of placental calcium or phosphorus transfer for each fetus in the litter can then be determined by expressing the accumulation of ^{45}Ca relative to ^{51}Cr or ^{32}P relative to ^{51}Cr, respectively. Strengths of this procedure include that the dam is awake, fetuses are not disturbed but remain in control of placental function, placental perfusion pressure has not been altered, and 8–10 normal and heterozygous fetuses within each litter serve as controls for the null fetuses and each other. The main limitation is that a relative and not absolute rate of mineral transfer among fetuses is obtained in order to be able to compare results from one experiment to the next.

3.2 Human Data

The calcium transporting cells in human placentas are the syncytiotrophoblasts and there is no structure equivalent to the rodent intraplacental yolk sac.[1] Human placentas are discoid and hemochorial but only a single layer of trophoblasts is interposed between maternal and fetal circulations, making them hemo*monochorial*. Finger-like projections (villi) protrude and float freely in a large intervillous maternal blood-filled space. The trophoblasts intensely express calbindin-D9k, Ca^{2+}-ATPase, and TRPV6. PTHrP mRNA and protein are widely expressed in human syncytiotrophoblasts,

cytotrophoblasts, amnion, decidua and myometrium; the highest levels may be in the amnion. Human trophoblasts also express the PTH1R, CaR, VDR, calcitonin, calcitonin receptor, NaPi2b, FGF receptors, and Klotho.[1]

Although in vitro perfusion studies have been done, no in vivo data about placental function have come from study of human placentas. Despite some differences in structure, hemochorial rodent placentas appear to have similar active transport and diffusion characteristics as hemochorial human placentas, whereas the epitheliochorial placentas of sheep differ significantly in structure and function from human placentas.

4 OVERVIEW OF ENDOCHONDRAL BONE DEVELOPMENT

4.1 Animal and Human Data

The details of endochondral bone development are beyond the scope of this chapter and therefore a brief overview is provided for context of what follows in later sections. The genetic control of endochondral bone development has been derived largely from studies in rodents, including various transgenic and knockout mouse models. The remainder of the data in this section largely derives from study of human embryos and fetuses that aborted or died in obstetrical accidents.

Studies in rodents have shown that early patterning of the embryonic skeleton is dependent upon a multitude of signaling pathways, which include Hox genes, Wnts, Hedgehogs, bone morphogenetic proteins, fibroblast growth factors, Notch/Delta, and other factors.[43,44] Mesenchymal cells are laid down in spatiotemporal patterns where bones of the axial and appendicular skeletons will form. These osteochondral progenitors differentiate into osteoblasts at the site of intramembranous bone formation, which includes the vault of the skull and a few other bones. In the bulk of the developing skeleton these progenitors differentiate into chondroblasts that initiate the process of endochondral bone development.

By the 8th week of gestation in humans, a complete cartilaginous scaffold of the skeleton is present with such fine details as digits and joints already delineated. The scaffold of each long bone lengthens at both ends, with the older cells nearer the center undergoing differentiation, hypertrophy, and then apoptosis. Chondroclasts and osteoclasts resorb the apoptosed chondrocytes and surrounding matrix, vascular invasion occurs, and osteoblasts lay down the primary spongiosa (osteoid) that will become mineralized. Primary ossification centers form in the vertebrae and long bones between the 8th and 12th weeks, but the bulk of mineralization does not occur until the 3rd trimester. Resorption and remodeling of bone to create secondary spongiosa occurs in utero and this resorption will abnormally increase when the fetus is stressed by maternal hypocalcemia. For example, uncontrolled hypocalcemia in a hypoparathyroid woman can lead to secondary hyperparathyroidism in the fetus, manifest by subperiosteal resorption, fractures, and reduced bone mineral content.[1] At the 34th week of gestation, secondary ossification centers form in the femurs, thereby creating true growth plates. But most epiphyses are cartilaginous until after birth when the secondary ossification centers finally form.[45] These growth plates become fused during puberty, after which linear growth ceases.

5 ROLE OF PTHrP

5.1 Animal Data

5.1.1 Regulation of Serum Minerals

PTHrP is present in human cord blood at levels up to 15-fold higher than PTH when expressed in equivalent pmol/L units, and similar high levels have been observed in fetal lambs and rodents.[1] Multiple fetal tissues produce PTHrP and so it is unclear from which source(s) PTHrP in the circulation derives. The placenta may be a dominant source because the normal placental expression of PTHrP is upregulated further in *Pthr1* null mice which have an 11-fold elevation in circulating PTHrP.[9] On the other hand there is conflicting evidence as to whether the fetal parathyroids contribute substantial amounts of PTHrP to the circulation. PTHrP was first detected in parathyroids of fetal sheep by using polyclonal antibodies. Since parathyroidectomy in fetal lambs causes hypocalcemia, but circulating PTH levels are normally low in fetuses, it was inferred that loss of parathyroid-derived PTHrP causes the hypocalcemia. However, no measurements of circulating PTHrP levels were made in these parathyroidectomized fetal lambs. In two subsequent studies PTHrP mRNA was not detected in fetal or adult rat parathyroids by in situ hybridization or real-time PCR (RT-PCR), whereas it was detected by RT-PCR in a third study of adult rat parathyroids.[1] Murine parathyroids have not been studied in isolation to determine if PTHrP mRNA or protein is present. However, absence of parathyroids in *Hoxa3* null and *Gcm2* null fetuses did not alter the plasma PTHrP level,[5,9] and the 11-fold upregulation in plasma PTHrP in *Pthr1* null mice was not associated with any detectable increase in expression of PTHrP mRNA in the neck region.[9] These data suggest that the parathyroids are not the main source of circulating PTHrP in fetal rodents.

PTHrP evidently regulates the fetal blood calcium because in its absence (*Pthrp* null fetuses) the blood calcium falls to the maternal level (Fig. 20.3). Though it does not fall even lower than this is due to a fivefold upregulation

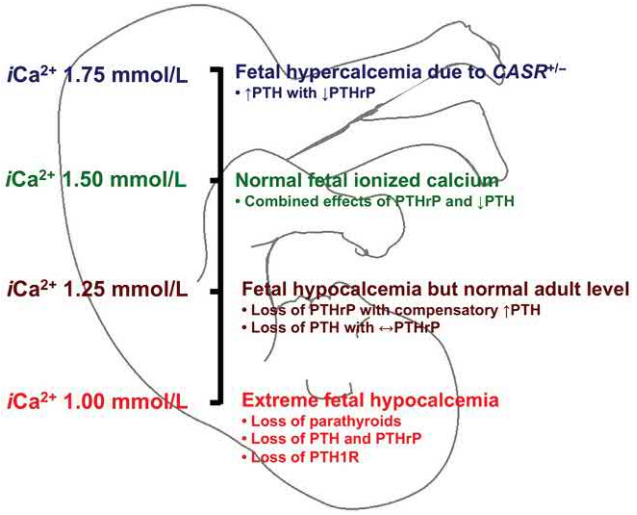

FIGURE 20.3 **Regulation of fetal ionized calcium.** This schematic illustrates changes in ionized calcium as observed in murine fetuses and extrapolated to humans; the levels shown are meant as a guide for relative changes and are not meant to indicated precise values found in human cord blood. The normal ionized calcium level is higher in fetuses than in the normal adult, and is maintained by the combined effects of high concentrations of PTH-related protein (*PTHrP*) and low levels of parathyroid hormone (*PTH*) in the circulation. CaSR likely suppresses PTH in response to the normal high level of calcium in fetal blood. The high fetal blood calcium may be set by a novel calcium-sensing receptor (other than the known CaSR), which regulates PTHrP release from the placenta and possibly other fetal tissues. In the absence of PTHrP, serum calcium falls but is now maintained at the normal adult level through the normal actions of CaSR to regulate PTH. Loss of PTH leads to a similar blood calcium value, approximately equal to the maternal calcium concentration, without a compensatory increase in circulating PTHrP. Combined loss of PTH and PTHrP, loss of parathyroids, or loss of PTH1R, each result in the lowest fetal ionized calcium values. Conversely, inactivating mutations of *CASR* cause PTH and the blood calcium to increase above normal fetal levels, in turn causing PTHrP to become decreased.

of circulating PTH, which is likely stimulated by CaR in order to maintain the normal adult level of blood calcium. The responsiveness of fetal PTH to regulation by CaR has been demonstrated in additional experiments. When CaR is simultaneously ablated (*Pthrp/Casr* double mutants) the fetal PTH and blood calcium rise higher than in *Pthrp* nulls.[3] On the other hand when the amino-terminal effector pathway for PTH and PTHrP are both removed (*Pthr1* nulls), the simultaneous ablation of CaR has no effect on the fetal blood calcium at all (*Pthr1/Casr* double mutants vs. *Pthr1* nulls) (Fig. 20.3).[3]

PTHrP also regulates phosphorus but not magnesium levels in blood because *Pthrp* null fetuses are hyperphosphatemic with normal serum magnesium concentrations.[8,9]

5.1.2 Regulation of Placental Mineral Transfer

PTHrP stimulates placental calcium transfer in fetal sheep and rodents. The original evidence (reviewed in Ref. 1) came from fetal sheep that were thyroparathyroidectomized and treated with thyroid hormone for several days before the fetus was removed and the in situ placental perfusion technique was performed. Placental calcium transfer was significantly reduced in this model but could be increased with infusion into the circuit of autologous blood from intact fetal sheep.[46,47] Additional experiments found that intact and midmolecular fragments of PTHrP each stimulated placental calcium transfer, whereas PTH was ineffective.[21,48–50] These results in isolated, perfused placentas of thyroparathyroidectomized fetal sheep are consistent with the concept that parathyroid-derived PTHrP regulates placental calcium transfer while PTH does not. However, circulating levels of PTHrP were not measured and so it remains unknown whether fetal thyroparathyroidectomy lowers the rate of placental calcium transfer by reducing fetal PTHrP.

Subsequent studies, using a placental calcium transfer technique in intact fetal mice, found that, depending upon the time point studied, *Pthrp* null fetuses had a 25%–40% reduction in the rate of placental calcium transfer as compared to their wt and $Pthrp^{+/-}$ littermates. Treatment with PTH and amino-terminal PTHrP had no effect, whereas intact and midmolecular PTHrP both raised the ^{45}Ca accumulation to normal.[14] These results confirmed that placental calcium transfer is stimulated by PTHrP but not PTH. However, *Pthrp* null fetuses have a threefold increase in endogenous PTH that may have blunted the ability of exogenous PTH to stimulate placental calcium transfer.

Additional studies in other knockout mouse models support the notion that PTHrP regulates placental calcium transfer while PTH may not. Aparathyroid *Hoxa3* null fetuses have undetectable circulating PTH[51] but a normal rate of placental calcium transfer, unaltered plasma PTHrP levels, and normal placental PTHrP expression.[9] *Pthr1* null fetuses have increased circulating PTHrP and PTH and a 50% higher relative rate of placental calcium transfer.[9,14] These *Pthr1* null fetuses are unable to respond to amino-terminal PTH or PTHrP but retain the ability of both hormones to act on any mid- or carboxy-terminal receptors. The high-circulating levels of PTHrP are thought to be stimulating placental calcium transfer through actions on a midmolecular receptor.[9,14]

Contrary evidence about the role of PTHrP comes from the in situ placental perfusion technique adapted for use in mice as described above. Perfused placentas from *Pthrp* null fetuses had an *increased* rate of ^{45}Ca transfer as compared to parallel studies done in wt and $Pthrp^{+/-}$ fetuses.[42] This surprising result may be explainable by the experimental methodology described earlier, most notably the use of nitroglycerin to dilate the placental and umbilical vasculature prior to catheterization of the umbilical vessels. PTHrP is expressed in the smooth muscle vasculature, functions as a smooth muscle relaxant and vasodilator, and exerts effects on vascular development and physiology.[52,53] The *Pthrp* null

placental vasculature likely differs from wt in its ambient pressure, structure, and responsiveness to vasodilators.[53] Consequently, nitroglycerin may have different effects on the vasculature of wt and *Pthrp* null placentas and lead to an artifactual result. Additional concerns about this technique have been detailed elsewhere.[1] Taken at face value, use of the miniaturized in situ placental perfusion technique suggests that PTHrP's role is to inhibit placental calcium transfer rather than to stimulate it. This finding, which is at odds with the bulk of the existing data, as noted above, may be artifactual due to the experimental methodology.

Overall most of the studies in fetal sheep and mice support the concept that a midmolecular form of PTHrP stimulates placental calcium transfer. Whereas the fetal sheep studies imply that the PTHrP is parathyroid derived, studies in aparathyroid fetal mice are more consistent with a placental source. Species differences might also explain the apparently discrepant results of the sheep and mouse studies.

Magnesium and phosphorus are also actively transported across the placenta but whether PTHrP plays a role is uncertain. Midmolecular fragments of PTHrP stimulated magnesium transport across in situ perfused placentas of fetal lambs [21,23] but had no effect in rat placentas.[54] PTHrP did not stimulate placental phosphorus transfer in sheep.[55,56]

5.1.3 Regulation of Endochondral Bone Formation

Within the developing endochondral skeleton, PTHrP is produced by perichondrium and proliferating chondrocytes while the PTH1R is expressed further down the growth plate within prehypertrophic chondrocytes,[57–59] as well as in preosteoblasts and osteoblasts.[60] The critical role that PTHrP plays in endochondral bone formation was first made evident by *Pthrp* null fetuses, which have a chondrodysplasia characterized by dwarfed long bones and deformed growth plates.[61] Further study has shown that PTHrP acts locally to delay terminal differentiation and hypertrophy of chondrocytes. Without PTHrP, hypertrophy begins early and bone formation starts before the cartilaginous template has reached its intended length; consequently, long bones become shorter than normal.[59,62] Conversely, overexpression of PTHrP or expression of an activating mutation of the PTH1R within fetal chondrocytes delays chondrocyte hypertrophy and results in a largely cartilaginous skeleton at birth.[63,64] PTHrP also interacts with Indian hedgehog and the wnt signaling pathway to regulate the lifespan and fate of chondrocytes.[59,65]

Since preosteoblasts and osteoblasts express PTHrP and PTH1R, the *Pthrp* null skeleton might be expected to show decreased expression in osteoblast-specific genes, and reduced bone formation and mineralization. However, *Pthrp* null growth plates normally express collagen α1(I) and collagenase-3, and have upregulated expression of osteocalcin and osteopontin.[61,66] The rapid maturation and apoptosis of the cartilaginous template within the *Pthrp* null skeleton causes bones to mineralize in utero that normally do not mineralize until after birth; moreover, the portions of the ribs and sternum which normally remain cartilaginous in the adult become bone in the *Pthrp* null fetus.[61] As noted earlier, *Pthrp* nulls have threefold increased circulating PTH[8] and this hyperparathyroidism may rescue any deficit in osteoblast function that loss of PTHrP might otherwise cause. Further studies in mice lacking PTH or the PTH1R support this notion (discussed later).

PTHrP expressed within preosteoblasts and osteoblasts does play an important role in regulating bone formation, as confirmed by a model in which the PTHrP gene deletion was targeted to these specific cells. Such mice have reduced bone formation and low bone mass at six weeks of age, confirming that osteoblast-derived PTHrP regulates bone formation.[60] However, the fetal skeletons have not been examined and so it remains unknown whether osteoblast-derived PTHrP is important for fetal bone formation.

As noted above, *Pthrp* null fetuses lack circulating PTHrP, are hypocalcemic (ionized calcium reduced to the maternal level), and have a reduced rate of placental calcium transfer.[8,14] Despite these changes and shorter long bones, the *Pthrp* null skeleton has a normal ash weight and normal content of calcium, magnesium, and phosphorus.[8,14] Why is skeletal mineral content not reduced? The *Pthrp* null skeleton accretes mineral more avidly than normal due to its accelerated and abnormal calcification of skeletal structures, and so its mineral content cannot be meaningfully compared to the normal skeleton.

Overall the evidence is that PTHrP participates in fetal skeletal development by upregulating placental calcium transfer, maintaining a high serum calcium, determining the fate of chondrocytes within the scaffold, and possibly by regulating preosteoblasts and osteoblasts.

5.2 Human Data

PTHrP is expressed by human placentas and may be a major contributor to the high-circulating concentration found in cord blood at term. Human fetal parathyroids have not been examined but PTHrP expression has been detected in adult normal, hyperplastic, and adenomatous parathyroids by Northern, immunohistochemical staining, immunoblot, and both radioimmuno and radioimmunometric assays.[1] A human equivalent of PTHrP gene deletion has not been identified but would presumably be lethal at or before birth if absence of PTHrP has consequences similar to those observed in fetal mice. A microdeletion in the PTHrP gene has been linked to brachydachtyly type E, a condition characterized by short

stature and shortened metacarpals and metatarsals.[67] This is consistent with a role for PTHrP in controlling endochondral bone formation in humans.

6 ROLE OF PTH

6.1 Animal Data

6.1.1 Regulation of Serum Minerals

Despite its low-circulating level in fetuses, PTH is an important regulator of the serum calcium. Loss of PTH, parathyroids, or PTH1R result in significant hypocalcemia as shown by *Pth* null, *Gcm2* null, *Hoxa3* null, and *Pthr1* null fetuses[5,9] and thyroparathyroidectomized fetal sheep. Blood calcium is lowest in fetal mice lacking parathyroids (*Hoxa3* null) or PTH1R (*Pthr1* nulls), whereas it is reduced only to the maternal level in mice lacking PTH (*Pth* nulls) (Fig. 20.3).

Although PTH upregulates in the absence of PTHrP, the converse is not true. Despite significant hypocalcemia, circulating PTHrP is normal in *Pth* null, *Gcm2* null, and aparathyroid *Hoxa3* null fetuses.[5,9] Circulating PTHrP is evidently not regulated by the fetal blood calcium and CaR, but may be responsive to local regulation within the placenta.

PTH regulates serum phosphorus as hyperphosphatemia occurs in thyroparathyroidectomized fetal lambs,[68,69] and in all PTH-deficient mouse models, including *Hoxa3* null, *Gcm2* null, and *Pth* null mouse fetuses.[5,9] Why the serum phosphorus is elevated is not known but decreased utilization of phosphorus for skeletal mineralization is one possible cause (discussed later).

Absence of PTH also leads to lower serum magnesium levels, most notably a 20% decline in aparathyroid *Hoxa3* null fetuses[9] but a much smaller decline in *Gcm2* nulls and *Pth* nulls.

Overall, despite low-circulating levels, PTH is an important regulator of serum calcium, phosphorus, and magnesium, because in its absence these mineral concentrations are reduced.

6.1.2 Regulation of Placental Mineral Transfer

Most studies have suggested that PTH does not regulate placental calcium transfer, and this includes the aforementioned placental studies in thyroparathyroidectomized fetal sheep and *Pthrp* null fetuses in which PTH was ineffective. However, other studies used decapitated fetal rats to simulate thyroparathyroidectomy and found that in situ perfused placentas had a reduced rate of calcium transfer that could be stimulated by exogenous PTH.[1] There was no effect of PTH in placentas from intact animals. The PTH1R is highly expressed within the intraplacental yolk sac,[38] and therefore well positioned to regulate placental mineral transport.

Studies of *Pth* null fetal mice (which completely lack PTH) and *Gcm2* null fetal mice (which have low levels of circulating PTH) have recently delineated a possible role for PTH in regulating placental cation transport.[5] Placental calcium transfer was normal in *Pth* nulls and the circulating PTHrP level was unchanged. However, microarray and RT-PCR analysis of *Pth* null placentas revealed reduced expression of several calciotropic genes (*Trpv6*, *Sg100, Vdr*) and other genes involved in cation and solute transport. To confirm that these changes in gene expression were due to absence of PTH, and to determine if PTH could stimulate placental calcium transfer, *Pth* null fetuses were treated in utero with either saline or full-length PTH before performing the placental calcium transfer experiment. *Pth* null fetuses treated with PTH had a 30% higher rate of ^{45}Ca accumulation as compared to *Pth* null fetuses treated with saline, confirming that PTH can stimulate placental calcium transfer in vivo.[5] PTH treatment also increased the placental expression of *Vdr*, vitamin D binding protein, and other solute carriers.[5]

In contrast to the *Pth* null phenotype, *Gcm2* nulls had a significantly increased rate of placental calcium transfer but no upregulation in PTHrP that could explain it.[5] *Gcm2* nulls also had significantly increased expression of placental *Trpv6*, *Sg100,* and *Vdr* as compared to *Pth* null placentas. *Gcm2* nulls and *Pth* nulls are similarly hypocalcemic and hyperphosphatemic, but only the *Gcm2* nulls upregulated placental calcium transfer and several genes known to be involved in calcium transport. The difference between the two models may be explained by expression of PTH within the placenta. PTH expression was increased in *Gcm2* null placentas and absent or near the limit of detectability in *Pth* null placentas.[5] Therefore, PTH may act locally to regulate placental calcium transfer independent of PTHrP.

Overall, placental expression of PTH and the PTH1R, stimulation of placental calcium transfer and gene expression in response to PTH injection in *Pth* null fetuses, and downregulation of placental genes in the absence of PTH, are all consistent with the conclusion that PTH acts locally in the placenta to regulate the transfer of calcium and other solutes. The effect of PTH may be weaker or conditional on activity of PTHrP, since *Pthrp* null fetuses have reduced placental calcium transfer despite threefold higher circulating PTH.

Experiments in fetal sheep suggest that PTH does not regulate phosphorus transfer[1] while there are no data on placental phosphorus or magnesium transfer in the various mouse models.

6.1.3 Regulation of Endochondral Bone Formation

There is mixed evidence for PTH's role in regulating endochondral bone formation and mineralization. Aparathyroid *Hoxa3* null fetuses in an outbred Black

Swiss background have low skeletal mineral content but otherwise show no abnormality of endochondral bone development or expression of osteoblast-specific genes, including collagen α1(I), collagenase-3, osteocalcin, and osteopontin.[8,9] In the same outbred background, double mutants lacking *Hoxa3* and *Pthrp* have an undermineralized form of the *Pthrp* null chondrodysplasia and are globally smaller than either single mutant.[8] These findings confirm that the chondrodysplasia seen in *Pthrp* nulls is largely due to absence of PTHrP and not the compensatory secondary hyperparathyroidism, while undermineralization of the skeleton results from loss of parathyroids and/or PTH.[8]

In contrast to the phenotype of aparathyroid *Hoxa3* fetuses in the Black Swiss background, *Pth* null fetuses in a C57BL/6 background have slightly shortened tibial metaphyseal lengths, shorter metacarpals and metatarsals, smaller vertebrae, reduced trabecular bone volumes, and fewer osteoclasts and osteoblasts.[11] Modest changes are also seen in expression of genes involved in chondrocyte maturation and apoptosis, mineralization, and vascular invasion of the growth plate.[11] Overall, the structural changes are largely in bone parameters, whereas the cartilaginous indices are little different from wt siblings. *Pth/Pthrp* double mutants are smaller and display an undermineralized form of *Pthrp* null chondrodysplasia (similar to the *Hoxa3/Pthrp* double mutants).[11] The data from this study indicate that PTH regulates bone formation in utero but not chondrocyte development.

A third study back-crossed *Pth* nulls and *Gcm2* nulls (which have little or no circulating PTH) from the original inbred C57BL/6 strains into outbred Black Swiss in order to compare to *Hoxa3* null fetuses.[5] Skeletons of both mutants are undermineralized but to a lesser extent than in *Hoxa3* null,[5,8] and there is no shortening of the long bones or alteration in trabecular bone volumes or skeletal morphology.[5] However, studies of gene expression within the *Pth* null and *Gcm2* null growth plates were not done. It is possible that alterations in serum calcium (which is approximately 0.25 mmol/L higher in Black Swiss[14]), or genetic differences in background strain, may explain why some alterations in bone parameters are seen in *Pth* null fetuses within one but not both background strains.

Overall the evidence indicates that PTH maintains the fetal blood calcium at a level sufficient to facilitate mineral accretion by the developing skeleton. Within developing endochondral bones PTH does not control chondrocytes, while its effects on osteoblast development vary with the genetic background or ambient serum calcium.

6.2 Human Data

The PTH1R is expressed within human placenta and PTH treatment has also been shown to increase ^{45}Ca accumulation in vitro within vesicles created from human syncytiotrophoblast basal membranes.[70] There have been no systematic measurements of fetal or cord blood mineral measurements from human fetuses that lack parathyroids or PTH, such as in DiGeorge syndrome. The animal data discussed above predict that fetal hypoparathyroidism will cause hypocalcemia and hyperphosphatemia in utero, as well as impaired skeletal mineralization.

7 ROLE OF PTHrP AND PTH IN COMBINATION

7.1 Animal Data

7.1.1 Regulation of Serum Minerals

Pthr1 null fetuses have the lowest ionized calcium level, equivalent to *Hoxa3/Pthrp* double mutants, and lower than in aparathyroid *Hoxa3* mutants (Fig. 20.3).[8,14] They are also hyperphosphatemic. PTH and PTHrP have additive roles in regulating the fetal blood calcium and skeletal mineralization since loss of both ligands or their common receptor leads to the greatest decline in blood calcium and skeletal mineral content.[8] However, it remains unclear why the ionized calcium is lower in aparathyroid *Hoxa3* null fetuses than it is in *Pth* or *Gcm2* null fetuses within the same genetic background.

7.1.2 Regulation of Placental Calcium Transfer

Placental calcium transfer is upregulated 50% in *Pthr1* null fetuses, presumably reflecting the 11-fold increase in systemic PTHrP and its actions on a midmolecular receptor.[8,14] This increase is relative to placental blood flow as inferred from the diffusion of ^{51}Cr-EDTA across the placenta. However the *absolute* amount of ^{45}Ca transferred to the *Pthr1* null fetuses is lower than in their wt littermates, in keeping with their much smaller size.[71]

7.1.3 Regulation of Endochondral Bone Formation

Pthr1 null fetuses are globally smaller than wt and display the *Pthrp* null phenotype of accelerated endochondral ossification and dysplasia combined with the *Hoxa3* or *Pth* null phenotype of significant undermineralization of the skeleton.[62] Whereas *Pthrp* null fetuses have normal expression of collagenase-3, upregulation of osteocalcin and osteopontin, and increased mineralization,[66] *Pthr1* fetuses have reduced expression of collagenase-3, osteocalcin, and osteopontin, and reduced mineralization.[66] This suggests that PTH upregulates expression of these osteoblast-specific genes in the *Pthrp* null, while blocking PTH action downregulates these genes in the *Pthr1* nulls. Moreover it indicates that, although placental delivery of mineral is relatively increased, this is insufficient for mineralization of the fetal skeleton. Thus the actions of PTH and PTHrP are needed to maintain the normally

high fetal blood calcium and stimulate osteoblast function and skeletal mineralization.

7.2 Human Data

Loss of the PTH1R in humans leads to Blomstrand chondrodysplasia, characterized by accelerated endochondral ossification and dysplasia that is similar to what has been seen in *Pthr1* null fetal mice.[72,73] It is normally lethal in utero; consequently, no measurements of serum calcium or phosphorus have been obtained.

8 ROLE OF ESTRADIOL

8.1 Animal and Human Data

The sex steroids circulate at low levels during fetal development and it is unclear whether they play essential roles in fetal calcium homeostasis and skeletal development. Mice lacking estrogen receptor alpha or beta, or the aromatase, have normal skeletal lengths at birth and do not develop altered skeletal metabolism until later.[74–76] However, none of these models have been examined during fetal development. Similarly human case reports have demonstrated that absence of the aromatase or the estrogen receptor lead to absent growth spurt, delayed bone maturation, failure of epiphyses to close, and continued growth into adulthood resulting in very tall stature. There are no fetal data beyond noting that birth weight, length, and early development were normal in a man who had inactivating mutations in the estrogen receptor.[77]

9 ROLE OF CALCITONIN

9.1 Animal and Human Data

Calcitonin appears unimportant for fetal and neonatal skeletal development since *Ctcgrp* null fetuses have normal placental calcium transfer, ionized calcium, calciotropic hormone levels, and endochondral bone development, gene expression and mineral content.[15] However, serum magnesium and skeletal magnesium content are both lower than normal, which suggests that calcitonin may regulate magnesium homeostasis.[15] In sharp contrast to this, absence of the calcitonin receptor causes embryonic lethality in mice,[78] a finding that remains unexplained. The calcitonin receptor may have critical roles independent of calcitonin during embryonic development; alternatively, maternal calcitonin may be able to rescue *Ctgrp* null embryos prior to establishment of the placental barrier.

There are no human data, as deletions of either the calcitonin or the calcitonin receptor gene have not yet been recognized in humans.

10 ROLE OF VITAMIN D AND CALCITRIOL

10.1 Animal Data

10.1.1 Regulation of Serum Minerals

Vitamin D and calcitriol are not required for fetal mineral homeostasis. The fetal blood calcium, phosphorus, and PTH remain normal despite severe vitamin D deficiency, ablation of vitamin D receptor, and absence of 1α-hydroxylase. The evidence comes from severely vitamin D–deficient rats,[16–18] *Cyp27b1* null pigs and mice [6,19] and both *Vdr* heterozygous and *Vdr* null mice.[4] These normal serum chemistries and PTH persist after birth until around the time of weaning, after which hypocalcemia, hypophosphatemia, and secondary hyperparathyroidism develop.

10.1.2 Regulation of Placental Mineral Transport

A few early studies demonstrated that pharmacological doses of calcitriol and other analogues could stimulate placental calcium transfer in fetal sheep and rats.[1] However, it appears that the placenta does not require calcitriol to pump calcium, as observed in studies of vitamin D–deficient rats,[79] *Vdr* null fetuses,[4,20] and *Cyp27b1* null fetuses.[6] Severely vitamin D–deficient rats show normal placental expression of calbindinD-9k and Ca^{2+}-ATPase, two factors involved in active placental calcium transfer.[79–81] *Vdr* null fetuses had *increased* placental calcium transfer, normal placental expression of calbindinD-9k and Ca^{2+}-ATPase, and upregulated expression of PTHrP and TRPV6.[4,20] The increase in placental calcium transport in *Vdr* null fetuses may mean that high levels of calcitriol are capable of stimulating placental calcium transport through receptor(s) other than the VDR. Since placental calcium transport is normal in severely vitamin D–deficient fetuses [79] and *Cy27b1* null fetuses,[6] calcitriol must not play a critical role in regulating placental mineral transport.

10.1.3 Regulation of Endochondral Bone Development

Not only the serum chemistries and PTH are normal at birth despite extreme disruptions in vitamin D/calcitriol physiology, but the skeleton is also normal. Severely vitamin D–deficient rats,[16–18] *Cyp27b1* null pigs and mice [6,19] and both *Vdr* heterozygous and *Vdr* null mice[4] have normal skeletal lengths, morphology, ash weight, and mineral content (determined both radiologically and by atomic absorption spectroscopic analysis of ashed bone). Normal, heterozygous, and *Vdr* null fetuses born of $Vdr^{+/-}$ mothers are indistinguishable from each other in all skeletal and biochemical parameters. Conversely heterozygous and null fetuses born of *Vdr* null mothers are smaller and weigh less than their counterparts from *Vdr*

heterozygous mothers, but have normal mineral content after adjustment for their smaller size.[4]

It is not until near the time of weaning that hypocalcemia, hypophosphatemia, secondary hyperparathyroidism, and rickets begin to develop in all of these models of disrupted vitamin D/calcitriol physiology.[16,17,19,82–85] This time course parallels a developmentally programed change of intestinal calcium absorption from a nonsaturable, passive process in the newborn to an active, saturable, calcitriol-dependent process.[1] Additional animal data have shown that calcitriol's role can be completely bypassed by initiating a calcium-enriched diet or parenteral calcium infusions at weaning, thereby maintaining a normal skeleton despite absence of vitamin D, vitamin D receptor, or 1α-hydroxylase.[86–88]

Collectively these findings indicate that vitamin D, calcitriol, or its receptor are not required for normal fetal calcium homeostasis, skeletal development, and mineralization. The animal studies predict that human babies born of vitamin D–replete and vitamin D–deficient mothers should be indistinguishable at birth in blood calcium, phosphorus, PTH, skeletal morphology, and mineral content.

10.2 Human Data

10.2.1 Observational Studies and Case Reports

The only systematic study that approaches the detailed analyses that have been done in animal models was published in 1925.[89] Babies that died of obstetrical accidents were examined radiologically and pathologically. The skeletal ash weight and mineral content (by atomic absorption spectroscopy) were indistinguishable between neonates born of normal mothers versus those born of mothers who had severe vitamin D deficiency and osteomalacia.[1] There also were no radiological signs of rickets and centers of ossification were normal.[89] The investigators had been convinced beforehand that they would find evidence of fetal rickets but admitted that they had found none.[89]

In contrast to this detailed analysis, occasional case reports and series have described that skeletal changes suggestive of rickets (usually craniotabes, ulnar cupping, or a widened fontanelle) can be detected at or soon after birth (studies cited in Ref. 1). Craniotabes is softening of the newborn skull bones that is subjectively assessed by palpating the skull; radiographs may suggest thinning of the skull bones or suture lines and fontanelles that are wider than normal. However, craniotabes has been determined to be a nonspecific finding that can be present in 30%–50% of healthy neonates, has no correlation with maternal or neonatal 25(OH)D levels or skeletal signs of rickets, and should not be used to indicate the presence of rickets or vitamin D deficiency.[90–95] Distal ulnar cupping is a normal variant that also should not be used to diagnose rickets.[96] A large or slowly closing anterior fontanelle can be seen with rickets,[97,98] but it is a nonspecific change shared by at least 25 metabolic, genetic, and skeletal disorders.[99,100] In fact, use of a widened fontanelle to presumptively diagnose vitamin D–deficient rickets led to misdiagnosis and maltreatment of a newborn with hypophosphatasia.[101]

In multiple reports that described craniotabes or rickets being present "at birth" the diagnosis was actually made within the first or second week, and so whether the fetus had any signs of rickets was not known.[1] In many cases where skeletal abnormalities suggestive of rickets were reported soon after birth, the mothers had significant malnutrition or anorexia, malabsorption (e.g., celiac disease, pancreatic insufficiency), or very low intakes of both calcium and vitamin D.[1] Consequently, the skeletal findings may not have been due to vitamin D deficiency alone but instead may have been the result of more global nutritional deficiency. This is also supported by the fact that in several reports the affected newborn hemorrhaged to death, an outcome that suggests disorders other than isolated vitamin D deficiency.[102]

The reported worldwide clinical experience with vitamin D deficiency is that hypocalcemia and rickets are not usually diagnosed until weeks to months after birth; rickets has a peak incidence between 6 and 18 months, even in regions where it is endemic.[1,103,104] Similar to the findings in rodents, studies in preterm and term human babies have shown that intestinal calcium absorption matures from a passive, nonsaturable process to an active, saturable, calcitriol-dependent process[1]; this likely explains why hypocalcemia, hypophosphatemia, and rickets do not usually develop until later in postnatal life.

At the extremes of abnormal vitamin D/calcitriol physiology, children with 1α-hydroxylase deficiency (vitamin D dependent rickets type I; VDDR-I) or those lacking the vitamin D receptor (vitamin D dependent rickets type II or hereditary vitamin D resistant rickets; VDDR-II) are also normal at birth.[1] In both conditions hypocalcemia, hypophosphatemia, and rickets eventually develop. Although both conditions can present soon after birth with biochemical abnormalities, VDDR-I usually presents late in the first year while VDDR-II is most often recognized in the second year or later.[1] It is possible that early biochemical abnormalities and rachitic changes have been overlooked in some of these cases, but the natural histories of VDDR-I and VDDR-II are quite compelling for their concurrence with the animal data. The impaired intestinal calcium absorption in VDDR-II patients can be bypassed by repeated calcium infusions or high oral dose calcium, thereby correcting the biochemical abnormalities and preventing or healing rickets.[1] Thus the human genetic disorders and the animal models provide coherent evidence that hypocalcemia and rickets are not present at birth but develop postnatally, and that the biochemical and skeletal abnormalities can be prevented

with administration of calcium alone. These data suggest that calcitriol's role in postnatal skeletal development is indirect through its stimulation of intestinal calcium absorption, whereas the intestines are not an important route of mineral delivery during fetal development.

Additional studies have examined bone mineral content in newborns.[1] In one study, forearm bone mineral content by 5 days of birth did not differ by use of vitamin D supplements during pregnancy.[90] In another, bone mineral content of the lumbar spine, femur, and whole body measured within 15 days after birth in 50 healthy term infants was not altered by vitamin D insufficiency versus sufficiency.[105]

10.2.2 Intervention Studies

More than a dozen randomized clinical trials of vitamin D supplementation during pregnancy have found no effect on cord blood calcium or phosphorus, anthropometric measurements, or radiological evidence of rickets.[1,2] These include a study of 126 babies where controls were severely vitamin D deficient [10 nmol/L 25(OH)D on cord blood], while babies from vitamin D–treated mothers had remarkably high-25(OH)D levels of 138 nmol/L.[106]

Among the few studies that enrolled mainly vitamin D–deficient women and achieved a substantial increase in 25(OH)D during pregnancy, Dawodu studied Arab women who had a mean 25(OH)D of 20.5 nmol/L at baseline, and were randomized to receive 400, 2000, or 4000 IU of vitamin D per day.[107] Achieved cord blood 25OHD levels at delivery were 40, 65, and 90 nmol/L, respectively.[107] No effect was seen on anthropometric parameters at birth or obstetrical outcomes.

Hollis and Wagner completed two studies that examined the effect of high-dose vitamin D supplementation during pregnancy in women who had much higher 25(OH)D levels at study entry. In the first study, 350 women (NCT00292591) randomized at 12–16 weeks of gestation received 400, 2000, or 4000 IU of vitamin D_3 per day,[108] achieved cord blood 25(OH)D was 46, 57, and 66 nmol/L, respectively. In the second study, 160 women (NCT00412087) randomized at 12–16 weeks received 2000 or 4000 IU of vitamin D_3 daily,[109] achieved cord blood 25(OH)D was 55 and 68 nmol/L, respectively. In both studies, apart from higher maternal and cord blood 25(OH)D levels, there was no effect on any obstetrical or neonatal outcome.[108–110] Several posthoc analyses of these two trials have subsequently been published, including analyses of selective pooled data from both studies, out of which some borderline significant results have been obtained.[108,110–113] However, these analyses suffer from lack of adjustment for multiple comparisons, arbitrary grouping of outcomes, double-counting of linked outcomes (preterm labor and preterm delivery), and exclusion of certain ethnicities from the analysis (discussed in Refs. 1,2). The first study's primary objective included bone mineral density of the newborns but this has not been reported.

Most recently, Cooper and Harvey provided preliminary results of the MAVIDOS study from the UK.[114,115] 900 women completed this after randomly being assigned to receive either 1000 IU of vitamin D or placebo beginning at 14 weeks of pregnancy. The study was revealed to be negative in its primary outcome (neonatal bone area, bone mineral content, and bone mineral density within the first 14 days after birth) and secondary outcome (anthropometric and body composition parameters within 48 h of birth).[114,115] A posthoc analysis, which was not prespecified in the clinical trial registrations (ISRCTN 82927713 and EUDRACT 2007-001716-23), suggested a possible benefit on bone mineral content of winter-born babies at 14 days of age. If the apparent benefit on winter-born babies is real and not a chance finding, it may reflect a postnatal effect of vitamin D supplementation during pregnancy, since the neonatal skeleton gains approximately 100 mg of calcium per day over the first 14 days after birth.[1,2] A measurement at 14 days does not indicate bone mineral content at term.

Overall these studies have largely examined the offspring of women who had baseline 25(OH)D values well above the 20 nmol/L threshold that has been shown to result in normal intestinal calcium absorption.[2,116–121] Few studies compared truly vitamin D deficient to vitamin D–replete women during pregnancy. It may take a larger study comparing those extremes to determine if there are differences in fetal or obstetrical outcomes, but ethical considerations may prevent such a study from being carried out in the modern day. The more recently published studies clearly establish the amount of maternal vitamin D intake that may be required to achieve various target levels of 25(OH)D in maternal and cord blood. However, none of these studies provide definitive evidence as to what 25(OH)D level might be optimal for bone health or nonskeletal health of the newborn. The overall results are consistent with the previously cited animal and human observational studies, in that isolated vitamin D deficiency is unlikely to alter serum minerals or skeletal development in utero. However, vitamin D deficiency will cause hypocalcemia and rachitic changes beginning sometime after birth, when the intestines become the main and calcitriol-dependent route of mineral delivery.

10.2.3 Associational Studies

In the last few years a number of associational studies have received significant attention in medical reviews and lay press, sparking concern that higher intakes of vitamin D are needed during pregnancy to insure optimal bone health at birth or later in life. These studies examined associations between single measurements of serum 25(OH)D during pregnancy in the mother and various skeletal outcomes in the fetus, neonate, or child.

In none of these any association was found with weight, skeletal lengths, or bone mineral density at birth.[122–127]

One study measured 25(OH)D at 28–32 weeks in 374 mothers and reported in a subanalysis that a 25(OH)D level below 28 nmol/L was associated with a slightly shorter knee-heel length in the offspring. However, although it was reported as a significant outcome, the difference was not statistically significant after correcting for gestational age.[122] Another study measured maternal 25(OH)D levels at 36 weeks in 424 women and found that 25(OH)D levels *below* 50 nmol/L were associated in the fetus with greater distal femoral metaphyseal cross-sectional area and a novel parameter called the "femoral splaying index."[123] The increased cross-sectional area of the femur was inferred by the authors to represent early rachitic change. In contrast, yet another study found that mothers *above* a median 25(OH)D level of 42.6 nmol/L had babies with a slightly greater tibial cross-sectional area.[124] The authors interpreted the increased cross-sectional area of the tibia to indicate stronger bone. These two studies exemplify how subjective the interpretation has been, with greater cross-sectional area of the metaphysis considered an adverse effect in one study and a benefit in another. Furthermore, the authors who reported a greater cross-sectional area of the tibia subsequently adapted their ultrasound technique to calculate femoral volumes in 357 mother–baby pairs.[127] Maternal 25(OH)D below 75 nmol/L was associated with slightly *reduced* femur length, width, and volume,[127] an association that is in the opposite direction from the original report of greater cross-sectional area of the tibia.[124] The seemingly contradictory results of these various studies could indicate that vitamin D/calcitriol has complex, site-specific effects on skeletal development. Alternatively, these could be chance findings with no true causal link between maternal 25(OH)D during pregnancy and the cross-sectional area or volume of fetal long bones.

A well publicized study by Javaid found no associations between maternal serum 25(OH)D and birth weight, length, placental weight, abdominal circumference, head circumference, or cord blood calcium.[125] At the 9 month follow up, there were still no associations of maternal 25(OH)D with skeletal and anthropometric parameters in the infants. However, at *9 years of age*, the investigators found a modestly lower bone mineral content in children whose mothers had had a serum 25(OH)D level below 27.5 nmol/L during pregnancy, as compared to children of mothers whose 25(OH)D levels had been 50 nmol/L or higher during pregnancy. These findings have been used to promote the theory that vitamin D exposure during fetal development programs childhood peak bone mass and adult risk of osteoporosis.[128] However, a recent study by Lawlor analyzed about 20 times the number of mother–child pairs than Javaid (3960 vs. 198), and found no associations between maternal 25(OH)D during pregnancy and offspring bone mass at age 9 years, as assessed by DXA.[126] Lawlor had about 8 times the number of women with 25(OH)D below 27.5 nmol/L (220 vs. 28 mothers), and therefore should have had greater power to detect an association if one were present.[126]

There are significant caveats about these associational studies. Why the tibia in one study and the femur in another, and not both sites, or more skeletal sites? The possibility of chance findings cannot be excluded, especially if more skeletal sites were examined but not reported. Moreover, these associational studies are confounded by factors which contribute to low maternal 25(OH)D levels, including maternal obesity, lower socioeconomic status, poorer nutrition, and lack of exercise, prenatal care or vitamin supplementation, etc. Therefore, is lower 25(OH)D simply a marker of a less healthy pregnant woman? In the long-term follow-up study by Javaid,[125] much time elapsed between birth (when no effect was seen) and 9 years of age (when lower bone mineral content was found). Did low 25(OH)D in utero program lower bone mineral content at 9 years of age, as the authors of that study contend? Or does the lower maternal 25(OH)D status in late pregnancy indicate lower socioeconomic status, poorer nutrition, obesity, and other factors in the mother that will remain unchanged over the next decade and will be shared with the child, thereby affecting growth and peak bone mass? Associational studies cannot distinguish between these possibilities nor can they prove causation.

Clinical trials are needed to control for the confounding and determine if increased vitamin D intake or supplementation during pregnancy, infancy, or childhood enables a higher peak bone mass to be achieved. To date a few trials of short duration have been done in infants, and either no effect or at best a transient effect on bone mass has been seen.[129–131]

Overall the available evidence (reviewed in more detail in Refs. 1,2) indicates that, in human babies, cord blood calcium, skeletal morphology, and skeletal mineral content, should all be normal at birth despite vitamin D deficiency or VDDR-I and VDDR-II. It is after birth that hypocalcemia and progressive rickets will eventually develop if these disorders remain untreated. The American Academy of Pediatrics and the Institute of Medicine both concluded that a target 25(OH)D blood level of 50 nmol/L is appropriate to optimize bone health for pediatric ages; there is no conclusive evidence that higher blood levels confer any additional benefit.[132]

11 ROLE OF FGF23

11.1 Animal Data

FGF23 plays an important role in regulating phosphorus and skeletal metabolism in the adult.[133,134] It inhibits

renal phosphate transporters (NaPi2a and NaPi2c) to cause renal phosphate wasting, reduces calcitriol by inhibiting Cyp27b1 and upregulating Cyp24a1, stimulates PTH, and reduces intestinal phosphorus absorption by reducing NaPi2b expression and lowering the concentration of calcitriol. In turn these effects influence endochondral bone development and mineralization.

But during fetal development FGF23 appears to not be required since the extremes of high-FGF23 concentrations (*Phex* or *Hyp* null), absence of FGF23 (*Fgf23* null), and absence of the FGF23 coreceptor Klotho (*Klotho* null), do not alter serum phosphorus, renal excretion of phosphorus into amniotic fluid, placental phosphorus transport, endochondral bone development, or skeletal content of calcium and phosphorus.[1,7,39,135] There were some minor changes in the renal and placental expression of genes relevant to phosphorus metabolism, including that renal *Cyp24a1* expression was reduced in *Fgf23* null kidneys, increased in *Phex* null kidneys, and unchanged in *Klotho* null kidneys, but these alterations in gene expression did not affect overall phosphorus and skeletal physiology.[1,7,39,135] It is evidently sometime after birth that FGF23 begins to exert its effects on the kidneys and bone metabolism.

11.2 Human Data

Very limited human data have found that intact FGF23 circulates at low values in cord blood, whereas the inactive C-terminal fragment circulates at twice the adult value.[29,136] Klotho is present in cord blood at sixfold adult and neonatal values, and it is expressed by human trophoblasts.[136] It is unclear whether these circulating concentrations imply relatively decreased, normal, or increased FGF23 activity in the fetal circulation.

There are no cord blood data from genetic disorders that cause excess or absence of FGF23. However, genetic loss of FGF23 (tumor calcinosis) has presented as early as 18 days after birth with hyperphosphatemia and a calcified mass.[137,138] In families with X-linked hypophosphatemic rickets, in which an inactivating mutation in *PHEX* leads to high levels of FGF23, serial observations have revealed that the serum phosphorus can become low during the second to sixth weeks after birth.[139]

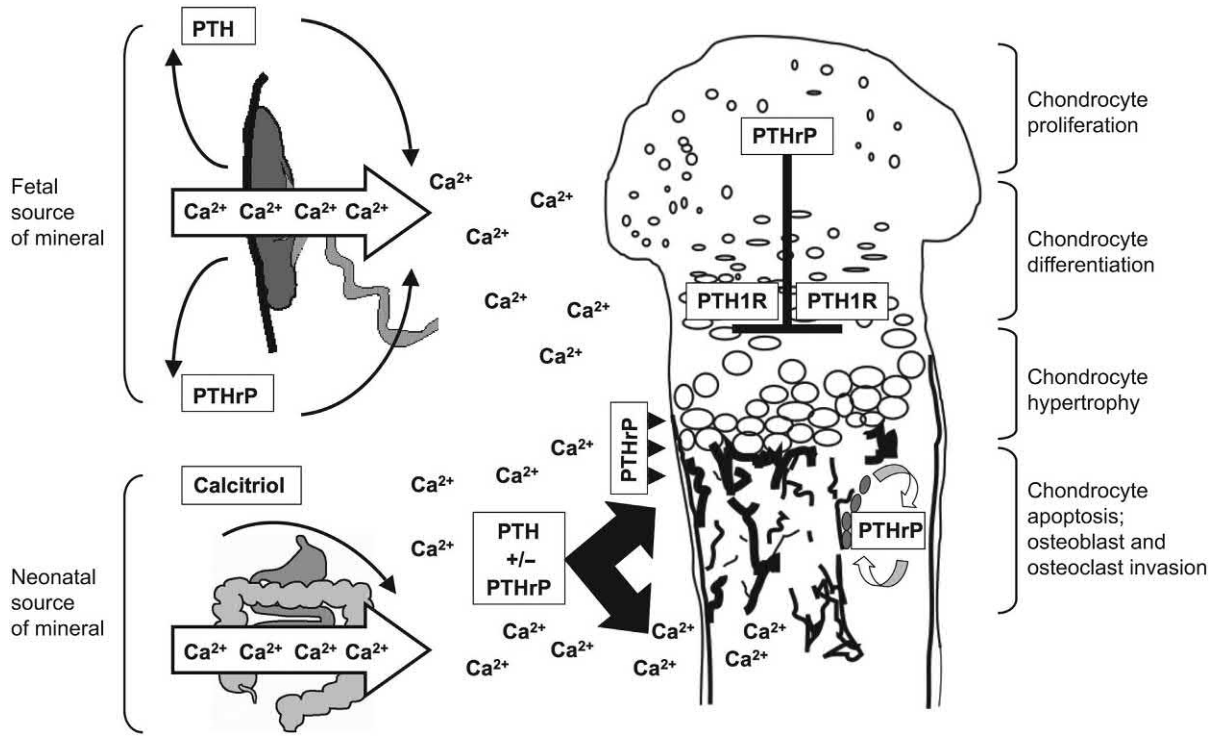

FIGURE 20.4 Relative roles of PTH, PTHrP, calcitriol, and FGF23 during fetal and neonatal life. The placenta is the main source of mineral during fetal life. PTH and PTHrP are expressed within the placenta but may also act on it from systemic sources to stimulate calcium transfer. The intestines are a trivial source of mineral in the fetus but are the main source for the neonate. Intestinal calcium absorption is initially passive but later becomes active, saturable, and calcitriol dependent in the infant. Within the endochondral skeleton, PTHrP is produced by proliferating chondrocytes and perichondrial cells (*arrowheads*) and delays terminal differentiation of prehypertrophic chondrocytes. PTHrP is also produced within preosteoblasts and osteoblasts and stimulates bone formation (*semicircular arrows*). During fetal life PTHrP and PTH act together to maintain high blood calcium and phosphorus levels in order to facilitate mineralization; loss of either PTHrP or PTH causes hypocalcemia and hyperphosphatemia. Calcitriol and FGF23 are not required to regulate serum minerals, endochondral bone formation, or skeletal mineralization in the fetus.

12 CONCLUSIONS

At all life stages adequate delivery of mineral is required for the skeleton to achieve and maintain appropriate mineral content and strength (Fig. 20.4). The placenta requires PTH and PTHrP but not calcitriol, FGF23, or calcitonin to regulate the pumping of mineral from the maternal circulation. Maintaining serum calcium and phosphorus at optimal levels for bone mineralization in utero requires PTH and PTHrP, but not calcitriol, FGF23, or calcitonin. Endochondral bone development in the fetus requires PTH and PTHrP but not calcitriol, FGF23, calcitonin, or (probably) the sex steroids. It is not until the later neonatal period, when intestinal calcium absorption becomes an active process, that skeletal development and mineralization begins to depend upon vitamin D/calcitriol. During the same interval, FGF23 begins to exert its effects on renal phosphorus handling and the synthesis and catabolism of calcitriol. The requirement for vitamin D/calcitriol can be bypassed by increasing the calcium content of the diet or by administering parenteral calcium infusions.

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CHAPTER

21

Control of Mineral and Skeletal Homeostasis During Pregnancy and Lactation

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1 INTRODUCTION

Normal pregnancy demands that women provide sufficient calcium to mineralize the fetal skeleton before birth, and in turn this necessitates several hormone-mediated adjustments in maternal mineral metabolism. Similarly, lactating women must supply sufficient calcium to breast milk to facilitate the nursing infant's continued skeletal growth and mineralization. The adjustments made in each of these reproductive periods differ significantly (Fig. 21.1). Intestinal calcium absorption upregulates during pregnancy whereas skeletal resorption predominates during lactation. The daily calcium needs of the fetus and infant are normally satisfied without requiring increased maternal intake of calcium or causing long-term adverse effects on the maternal skeleton. In turn these adaptations can affect the presentation and management of disorders of mineral metabolism during pregnancy and lactation.

Our knowledge of mineral homeostasis during pregnancy and lactation comes from surgical, pharmacological, and, more recently, genetically engineered animal models. Observational studies and clinical trials have clarified some of the areas where humans and animal models have the same or different adaptations to the challenges of pregnancy and lactation. This chapter will review both animal and human data and clarify where and if the findings differ. To avoid exceeding specified limits on the reference list, the reader is directed to two longer comprehensive reviews that cite the original research papers.[2,3]

2 SKELETAL AND MINERAL PHYSIOLOGY DURING PREGNANCY

The developing fetus requires only a small amount of mineral during the first two-thirds of gestation. After that time the placenta upregulates expression of calbindin-D9, Ca^{2+}-ATPase, transient receptor potential vanilloid 6 (TRPV6), and other genes that may be involved in calcium transport.[3] Active pumping of calcium from the maternal to the fetal circulation then begins and is followed by rapid mineralization of the fetal skeleton. Fetal rats have about 12 mg of calcium at birth of which 95% is accreted during the last 5 days of their 22-day gestation[4]; term fetal mice have about 1.5–2.0 mg of calcium with most of that accreted during the last 4 days of a 19-day gestation.[3] Conversely the human skeleton accretes about 30 g of calcium, 20 mg of phosphorus, and 0.8 mg of magnesium by term, with 80% of each mineral accreted during the third trimester.[4–11] The rate of net calcium transfer ranges from 60 mg per day at week 24 to 300–350 mg per day during the final 6 weeks of pregnancy.[2]

2.1 Mineral Ions and Calciotropic Hormones (Fig. 21.2)

Vascular fluid volume increases during pregnancy, which causes a dilutional fall in serum albumin and with it, a fall in total serum calcium. However, both the ionized calcium (the physiologically important fraction) and the albumin-corrected serum calcium remain unchanged throughout pregnancy in longitudinal studies of humans and some mouse strains.[2] In other animal

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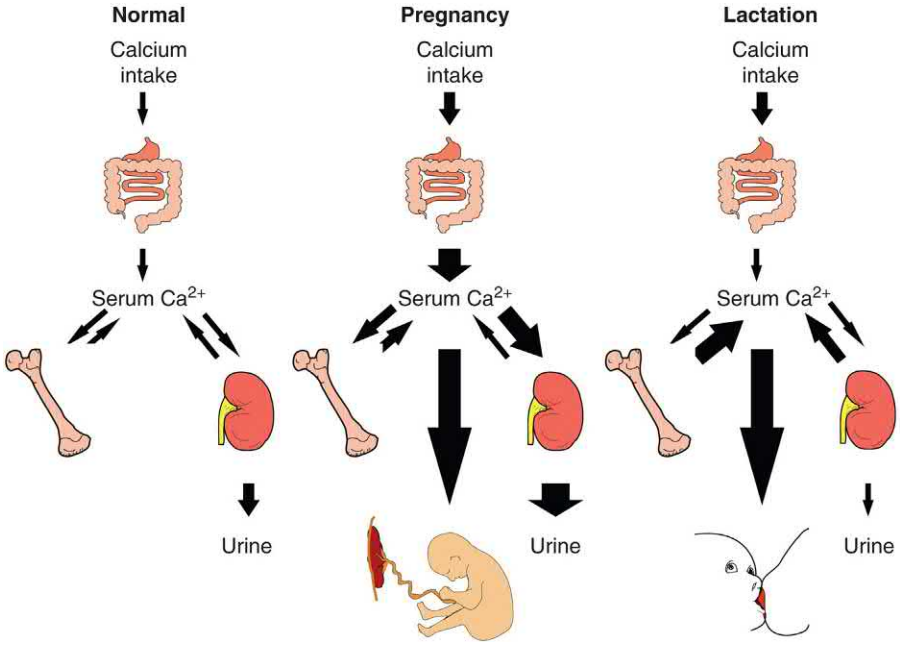


FIGURE 21.1 Schematic illustration contrasting calcium homeostasis in human pregnancy and lactation, as compared to normal. The thickness of arrows indicates a relative increase or decrease with respect to the normal and nonpregnant state. Although not illustrated, the serum (total) calcium is decreased during pregnancy, while the ionized calcium remains normal during both pregnancy and lactation. *Source: Adapted from Kovacs CS, Kronenberg HM. Maternal-fetal calcium and bone metabolism during pregnancy, puerperium, and lactation.* Endocr Rev *1997;***18***(6):832–72, copyright 1997, The Endocrine Society.*[1]

models, the mother's ability to regulate her blood calcium may become overwhelmed by the peak fetal demands during late pregnancy. Consequently, the ionized calcium may fall during late pregnancy in rats, deer, and sheep; moreover, larger litter sizes in the rat are associated with a greater decrease in maternal blood calcium.[2]

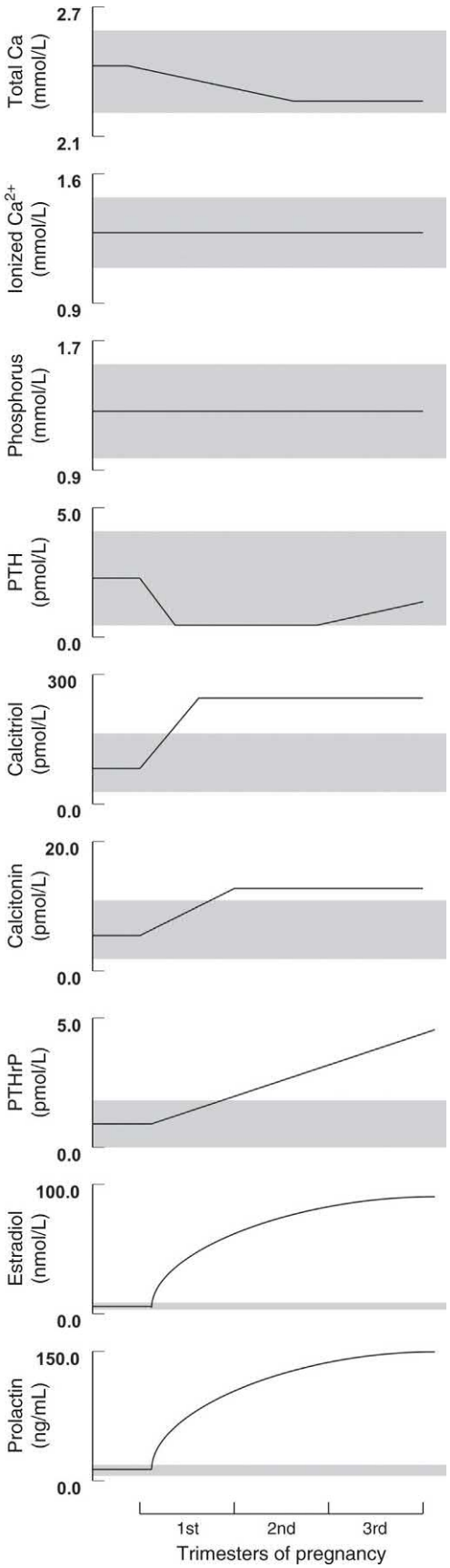
Serum phosphorus and magnesium levels are normal throughout pregnancy in humans and other animals.[2] Urine phosphorus levels decrease in mice,[12] likely due to suppressed parathyroid hormone (PTH).

The older human and animal literature pertaining to PTH values during pregnancy may be confusing. Assays from the 1970s and 80s used polyclonal antibodies that measured biologically inactive C-terminal or N-terminal fragments of the hormone which accumulate during pregnancy. The apparently high PTH levels based on these assays prompted the conclusion that physiological hyperparathyroidism occurs during pregnancy in humans and other animals; however, this appears to be incorrect. With newer "intact" PTH assays, most studies of women from North America and Europe (who generally have a calcium-sufficient diet) have shown that PTH falls toward the lower end of the normal range during the first trimester before increasing to the midnormal range by term.[13–17] In contrast, studies of women from Asia and Africa found that the PTH value does not become suppressed and may exceed the normal range in some individuals.[2,18] These women typically have diets that are low in calcium and high in phytate (which blocks calcium absorption), and that may explain why their PTH levels do not decrease.

Animal data that arise from use of the newer PTH assays remain somewhat variable, influenced by the calcium content of the diet, litter size, and species or strain.[2] Serial measurements in pregnant mice have found that PTH falls to about 20% of the nonpregnant value, with many individual values becoming undetectable.[12,19–21] Consumption of a high-calcium diet results in even lower PTH concentrations.[22,23] In contrast, measurements in pregnant rats have found decreased, no change, or increased PTH; the most common finding is increased PTH accompanied by reduced ionized or albumin-corrected serum calcium.[2] PTH rises earlier and to a higher level in pregnant rats with larger litters or when the calcium content of the diet is reduced.[2]

Overall the available animal and human data suggest that serum PTH normally becomes suppressed during pregnancy in humans and mice unless dietary calcium is insufficient, whereas secondary hyperparathyroidism is often observed in pregnant rats despite a calcium-sufficient diet.

In humans and rodents, total calcitriol levels increase two- to fivefold beginning early in pregnancy and maintain this increase until term; the free level is also increased during all three trimesters.[2] PTH is normally the major stimulator of the renal 1α-hydroxylase, and is critically



required to maintain calcitriol within the normal range of nonpregnant animals and humans. However, during pregnancy in humans and mice, PTH typically declines while calcitriol increases markedly.[2] Moreover a marked pregnancy-related increase in calcitriol occurs despite loss of PTH in *Pth* null mice,[21] parathyroidectomized rats,[24] and parathyroidectomized sheep.[25] These data indicate that calcitriol's rise during pregnancy is not driven by PTH. Instead, there is evidence that PTH-related protein (PTHrP), estradiol (E_2), prolactin (PRL), placental lactogen, and other factors may contribute to upregulation of the renal 1α-hydroxylase during pregnancy.[2]

The source of increased calcitriol production is largely the maternal kidneys, which show a two- to fivefold upregulation in renal 1α-hydroxylase during pregnancy.[2] However, some evidence in pregnant rats indicates that the placenta, fetus, and other maternal tissues may be additional sources of calcitriol due to expression of 1α-hydroxylase. When tritiated 25-hydroxyvitamin D was administered to totally nephrectomized rats, no tritiated calcitriol appeared in nonpregnant dams whereas some tritiated calcitriol (much reduced compared to normal) appeared in pregnant dams.[26,27] Those studies demonstrated that placenta and extrarenal maternal tissues can form calcitriol, but not to the high levels normally achieved during pregnancy. Additional studies in 5/6-nephrectomized rats found high PTH and a 40% reduction in calcitriol in virgin animals, whereas during pregnancy, PTH suppressed to normal and calcitriol increased to the high values normally reached during pregnancy.[28] Rather than proving that extrarenal sources of 1α-hydroxylase account for the normal pregnancy-related increase in calcitriol (as the authors concluded), that study may simply have demonstrated the ability of the remnant kidney to upregulate 1α-hydroxylase in response to the hormonal milieu of pregnancy. After all, the 5/6-nephrectomy model causes only a 50% reduction in creatinine clearance[29] and, as noted above, the pregnancy-related rise in calcitriol was markedly reduced in completely nephrectomized rats.[26,27] More compelling data from 1α-hydroxylase null pigs and *Cyp27b1* null mice suggest that fetus and placenta do not contribute a significant amount of calcitriol to the maternal circulation. Maternal

FIGURE 21.2 Schematic depiction of longitudinal changes in calcium, phosphorus, and calciotropic hormone levels during human pregnancy. Shaded regions depict the approximate normal ranges. Parathyroid hormone (PTH) does not decline in women with low calcium or high phytate intakes, and may even rise above normal. Calcidiol (25OHD) values are not depicted; most longitudinal studies indicate that the levels are unchanged by pregnancy, but may vary due to seasonal variation in sunlight exposure and changes in vitamin D intake. Fibroblast growth factor-23 (FGF23) values cannot be plotted due to lack of data. *Source: Reproduced with permission from Kovacs CS. Maternal mineral and bone metabolism during pregnancy, lactation, and post-weaning recovery.* Physiol Rev *2016;***96***(2):449–547.*[2]

calcitriol was very low to undetectable and comparable to nonpregnant values in these animals that cannot make calcitriol, even though they bore heterozygous placentas and fetuses that could make calcitriol.[23,30] Limited human data come from rare reports of anephric women who had successful pregnancies; in one published report, calcitriol was very low before and during pregnancy, confirming that the pregnancy-related increase in calcitriol is abolished in the absence of maternal kidneys.[31] Overall, while the anephric rat data indicate that extrarenal production contributes some calcitriol to the maternal circulation during pregnancy, it appears likely that, in women and rodents, most of the calcitriol comes from upregulated synthesis in the maternal kidneys.[2]

Serum calcitonin (CT) levels increase during pregnancy and may derive from synthesis within the thyroid, mammary tissue, and placenta.[2] Thyroidectomized women had undetectable CT levels when nonpregnant, but achieved CT levels during pregnancy that were higher than those in nonpregnant women with intact thyroids.[32]

Circulating PTHrP increases during pregnancy and may derive from multiple maternal and fetal tissues, with mammary tissue and placenta being the most likely sources.[2] PTHrP's rise in the maternal circulation does not occur until later in pregnancy and so it is unlikely to explain the early rise in calcitriol. Moreover, there is an evidence that PTHrP's different receptor binding, signaling characteristics, and crystal structure may make it less potent than PTH at stimulating the renal 1α-hydroxylase.[33–36] Whether circulating PTHrP plays a role in maternal calcium homeostasis of pregnancy is unclear, although it has been suggested that the carboxyl-terminal portion of PTHrP (osteostatin) may protect the maternal skeleton from excessive bone resorption.[2] Placental calcium transport is regulated by PTHrP in the fetal circulation, but it is not known whether PTHrP in the maternal circulation plays a role.[2,37]

Fibroblast growth factor-23 (FGF23) is a more recently recognized as a regulator of phosphorus homeostasis but it has not been extensively studied during pregnancy. There are as such no published data on FGF23 levels during human pregnancy; one study found that intact FGF23 values at 24 h after delivery were similar to nonpregnant controls.[38] Serial studies in mice have shown that intact FGF23 increases twofold during pregnancy.[21,39] The physiological significance of increased FGF23 in pregnancy is unclear as serum phosphorus remains unchanged. Moreover, although FGF23 reduces serum calcitriol in nonpregnant *Phex (Hyp)*$^{+/-}$ mice[40] and in *Fgf23* overexpressing mice,[41] serum calcitriol was no different among pregnant mice that had high (*Phex*$^{+/-}$), normal (WT), or reduced (*Fgf23*$^{+/-}$) concentrations of FGF23.[42,43]

PRL and placental lactogen both increase 10 to as much as 100-fold during pregnancy and activate PRL receptors.[2] As osteoblasts express PRL receptors, and PRL receptor-deficient mice show decreased bone formation, PRL or placental lactogen conceivably regulate skeletal metabolism during pregnancy. However, this possibility has not been rigorously studied. A single study manipulated the PRL level in pregnant rats by use of bromocriptine, and found that it blunted a pregnancy-related gain in bone mineral content.[44]

Circulating oxytocin (OT) levels rise as pregnancy progresses.[45] OT's classic role is to stimulate contractions of the uterine muscle, thereby facilitating delivery of the fetus and placenta. But the OT receptor is expressed by osteoclasts and osteoblasts[46] and so OT may also regulate bone metabolism during reproduction. Both male and female mice lacking OT or its receptor have an osteoporotic phenotype characterized by low bone formation.[47] Additional studies have shown that OT stimulates osteoblast differentiation and function, stimulates osteoclast formation, but inhibits osteoclast function and skeletal resorption.[47,48] Whether OT is specifically important for maternal bone metabolism during pregnancy is unclear because it has not been directly studied in vivo.

The production of numerous other hormones increases during pregnancy, including 100-fold increases in E_2, and lesser increases in estrone, estriol, leptin, placental growth hormone, and IGF-1.[2] Pituitary release of growth hormone decreases.[2] The extent to which these play a role in regulating intestinal calcium absorption or skeletal metabolism during pregnancy is unknown.

2.2 Intestinal Absorption of Calcium

Intestinal calcium absorption more than doubles during pregnancy and this appears to be the main mechanism that enables the fetal calcium demand to be met.[2] Placental phosphorus and magnesium absorption are similarly increased.[2] Studies using stable isotopes of calcium and other methods have revealed that intestinal calcium absorption is upregulated by midpregnancy in rodents and 12 weeks of gestation in humans; the most marked increase is late in pregnancy in both species, corresponding to the interval of greatest fetal demand for mineral.[2] A positive calcium balance results by midpregnancy in rodents and humans, and skeletal mineral content may also increase.[20,49] Isotope studies in rats have revealed that 92% of fetal skeletal mineral content derives from the maternal diet during pregnancy.[50]

As total calcitriol levels double or triple during pregnancy, it has been assumed that calcitriol regulates the increased intestinal calcium absorption. However, studies in rodents indicate that a pregnancy-related increase in intestinal calcium absorption precedes the rise in calcitriol and occurs despite severe vitamin D deficiency,[51,52] absence of the vitamin D receptor,[22] or maternal parathyroidectomy.[53] This upregulated calcium absorption is physiologically significant because

vitamin D-deficient rats and *Vdr* null mice both achieved a significant increase in skeletal mineral content during pregnancy, which reached 155% of baseline in *Vdr* nulls.[2,22] In humans intestinal calcium absorption doubles from the first trimester and may precede an increase in free calcitriol.[2] These data suggest that factors other than calcitriol must also stimulate intestinal calcium absorption during pregnancy.

Additional animal studies suggest that the *Vdr* null mouse compensates by upregulating intestinal expression of the calcium channel TRPV6,[22] and that PRL and placental lactogen may stimulate intestinal calcium absorption independently of calcitriol, possibly by stimulating TRPV6.[54–58] Although compelling animal data indicate that calcitriol or its receptor are not required to upregulate intestinal calcium absorption during pregnancy, no studies have examined the effects of vitamin D deficiency on intestinal calcium absorption during human pregnancy. A clinical study found that hyperprolactinemic men and women had calcitriol levels and rates of intestinal calcium absorption that were similar to normoprolactinemic controls[59]; however, the hormonal milieu of hyperprolactinemia differs from that of pregnancy, especially with respect to the relative concentrations of sex steroids.

2.3 Renal Handling of Calcium

In rats and mice a doubling of urine calcium excretion may be present by the second week of gestation.[2,12] In humans the 24-h urine calcium excretion increases from as early as the 12th week and hypercalciuric values are often present, whereas the fasting urine calcium is typically normal or low.[2] Random, nonfasted spot urine samples do not show this increase.[2] Hypercalciuria occurs because of increased intestinal calcium delivery (absorptive hypercalciuria) combined with the effects of suppressed PTH and increased CT to reduce reabsorption of calcium by the renal tubules. Hypercalciuria contributes to an increased risk of kidney stones during pregnancy, and also indicates that many women consume more calcium than needed during pregnancy.

2.4 Skeletal Calcium Metabolism

The skeleton contains over 99% of calcium in the body and represents a substantial reserve that will be borrowed from when dietary intake of calcium is insufficient. The fetus demands a substantial amount of calcium late in pregnancy and this leads to the concern that pregnancy may cause excessive skeletal resorption, low bone mass, and fractures. On the other hand, as intestinal calcium absorption doubles at beginning early in pregnancy, one might predict that bone mineral content will be increased prior to the peak fetal demands for calcium.

Studies in animals have shown that bone turnover increases during pregnancy but this leads to changes in skeletal structure and mineral content, which vary markedly within and between species.[2] Pregnant rats have histomorphometric evidence of increased bone formation and resorption but usually no net change in skeletal mass, mineral content, or bone strength at the end of pregnancy.[2] Those cross-sectional studies examined rats at the beginning versus the end of pregnancy and therefore did not determine whether there were interim changes in bone mass or mineral content at midpregnancy. Cortical bone volumes increase as a result of periosteal bone formation during pregnancy in rats, possibly as an adaptive response to the increasing body weight.[60,61] In mice, bone turnover increases as suggested by consistently higher levels of bone formation [procollagen I N-telopeptides (P1NP) and osteocalcin] and resorption markers [deoxypyridinoline and C-telopeptide (CTX)].[12,20,22] However, the net effect that increased turnover has on skeletal mass and mineral content of wild-type mice varies by skeletal site and genetic background. Serial studies in outbred Black Swiss mice show a progressive gain of 15%–20% in whole body bone mineral content during pregnancy but no change in the spine or hindlimb.[20,22] Conversely, inbred C57BL/6 mice lose about 5% of whole body bone mineral content and 15% of spine bone mineral content but gain more than 5% in the hind limb,[12] while CD-1 mice show no significant changes in mineral content at any skeletal site during pregnancy.[2,62,63]

The only histomorphometric data from women found that bone resorption parameters were higher at 8–10 weeks in 15 women who electively terminated pregnancy as compared to pregnant women at term and nonpregnant controls; however, poor age matching, small sample sizes, and confounding may have led to spurious results.[64] Additional studies have measured bone formation and resorption markers and inferred that bone turnover increases during pregnancy.[2] These studies share a number of confounding problems, including lack of prepregnancy baseline values (comparison to nonpregnant controls or normal ranges); effects of hemodilution on serum markers; increased globular filterate rate and altered creatinine excretion affecting urine values; possible contribution from placental, uterine and fetal sources; degradation and clearance by the placenta; and lack of diurnally timed or fasted specimens. Twenty four hour urine collections eliminate the need for creatinine values and are likely more reliable than spot urine samples during pregnancy. Overall the studies have reported that several markers of bone resorption (urine N-telopetide or CTX, serum CTX, tartrate-resistant alkaline phosphatase, deoxypyridinoline/creatinine, pyridinoline/creatinine, and hydroxyproline/creatinine) are increased from early to midpregnancy onward. Serum markers of bone

formation (osteocalcin, procollagen I carboxypeptides, and bone specific alkaline phosphatase) are often lower in early or midpregnancy as compared to prepregnancy values or normal adult ranges, but increase to normal or above before term.[2] Total alkaline phosphatase increases mainly due to placental production and is not a useful marker of bone formation during pregnancy.

The scant bone biopsy data and the bone biomarkers suggest that bone turnover increases from as early as the 10th week of gestation, but whether this leads to increased, decreased, or no change in bone mass during human pregnancy remains uncertain. Women are in a positive calcium balance by midpregnancy[49] with comparatively little maternal–fetal calcium transfer occurring until the third trimester, and so one might expect that skeletal mineral content should be increased at that time point.

Skeletal calcium content has been assessed in longitudinal studies that measured areal bone mineral density (aBMD).[2] Concerns about fetal radiation exposure have severely limited the data, and the studies are also confounded by changes in body composition (fluid and fat mass), weight, and skeletal volumes, which in turn may cause artifactual changes in the aBMD reading. Early studies used single or dual-photon absorptiometry to study the distal forearm and femur, respectively, and no significant change was found in cortical or trabecular aBMD during pregnancy or between pre- and postpregnancy measurements.[2] More recent studies have used dual X-ray absorptiometry (DXA) which has better precision, but most studies have been quite small with 16 or fewer subjects. Zero change in lumbar spine aBMD was seen in one study but losses of up to 5% of lumbar spine BMD occurred in the remaining studies.[2] However, these studies relied on measurements done up to 18 months before planned conception and 1–6 weeks after delivery with no measurements obtained during pregnancy.[2] Notably the postpartum period is associated with bone density losses of 1%–3% per month (see Section 4) and measurements obtained by 6 weeks postpartum will be confounded by lactational losses.

The largest study involved 92 pregnant Danish women who had DXA measurements of hip, spine, and radius done at baseline (up to 8 months prior to pregnancy), DXA of the forearm repeated during each trimester, and DXA of hip, spine, and radius done again 15 days after delivery. Seventy three women completed the postpartum visit. Compared with 57 controls who completed the same interval of follow up, BMD decreased in pregnant women by 1.8% at the lumbar spine, 3.2% at the total hip, 2.4% at the whole body, and 4% at the ultradistal radius, but increased by 0.5% at the proximal one-third forearm.[65] At the total forearm a 1% difference between pregnant and control became apparent at the third trimester measurement, coinciding with the peak interval of placental–fetal calcium transfer. Apparently all of the pregnancy cohort breastfed, and so lactation-induced bone loss confounded the measurements at day 15 postpartum.

Other longitudinal studies have used serial ultrasound measurements of the os calcis or phalanges and found small decreases during pregnancy in values that correlate with volumetric bone density.[2] However, the significance of changes at these sites is uncertain as compared to the more clinically relevant spine, hip, and radius. Moreover, it is concerning that ultrasound doesn't show any decline in apparent BMD of the os calcis during lactation despite significant losses being detected by DXA at spine and hip[66] (see Section 4). It remains to be shown if this relates to technical differences or site-specific differences in skeletal responses.

Overall, DXA and ultrasound studies lack serial measurements at spine or hip during pregnancy and cannot determine whether aBMD actually increases earlier in pregnancy as suggested by calcium balance studies. The results do suggest that transient declines in skeletal mineral content may occur during normal pregnancy, and can be statistically significant when mean changes in a cohort are analyzed. However, the magnitude of these changes is small and not likely to be detectable or considered significant in an individual subject.

It appears certain that pregnancy causes no long-term changes in skeletal calcium content or strength. There have been many dozens of epidemiological studies of young and older women that have failed to find significant associations of parity (or breast feeding) with bone density or fracture risk.[2,67] Moreover, adolescent pregnancy does not reduce peak bone mass as previously feared. In an NHANES III analysis of 819 women aged 20–25, women who had been pregnant as adolescents had the same BMD as nulliparous women and women who had been pregnant as adults.[68] However, these data do not exclude that transient but clinically significant bone loss might occur during pregnancy in some women; as noted below, more substantial but transient bone loss occurs in most women during lactation.

3 DISORDERS OF BONE AND MINERAL METABOLISM DURING PREGNANCY

3.1 Osteoporosis in Pregnancy

There are no animal models that suggest that osteoporotic fractures may occur as a consequence of normal pregnancy. However, women occasionally present with vertebral compression fractures during pregnancy and may be found to have low BMD shortly afterward.[69] In most cases, lack of baseline data means that low bone density or skeletal fragility preceding pregnancy cannot

be excluded. Some women may experience excessive resorption of calcium from the skeleton due to changes in mineral metabolism induced by pregnancy, and aggravated by factors, such as low dietary calcium and vitamin D deficiency.[69] A high rate of bone turnover is an independent risk factor for fragility fractures in nonpregnant individuals, and so increased bone turnover during normal pregnancy may increase fracture risk. So too the higher maternal weight of pregnancy and anterior position of the gravid uterus increase spine loading and may predispose to crush fractures. Consequently, fragility fractures during or shortly after pregnancy may be the result of preexisting low bone density and fragility disorders, increased bone resorption during pregnancy, higher body weight, and other factors.[69] However, when bone loss does occur, it appears to be transient and without conferring long-term fragility, as suggested by the epidemiological studies mentioned earlier.

Another pregnancy-related disorder is focal, transient osteoporosis of the hip.[69] This is likely not the result of altered mineral and bone metabolism during pregnancy, but instead may be a consequence of local factors. Proposed theories to explain this condition have included femoral venous stasis due to pressure from the pregnant uterus, a form of Sudeck's atrophy or reflex sympathetic dystrophy (causalgia), ischemia, trauma, viral infections, marrow hypertrophy, immobilization, and fetal pressure on the obturator nerve. These patients have unilateral or bilateral hip pain, limp and/or hip fracture in the third trimester or early postpartum. MRI reveals increased water content of the femoral head and the marrow cavity that likely contributes to an artifactually low aBMD by DXA; a joint effusion may also be present. The symptoms and the radiological appearance usually resolve within 2–6 months postpartum.

3.2 Primary Hyperparathyroidism

No animal models of primary hyperparathyroidism have been studied during pregnancy, although both acute and chronic maternal hypercalcemia have been found to suppress the fetal PTH level,[2,70] potentially contributing to neonatal hypoparathyroidism.

The human condition is uncommon with several hundred cases alluded to in the literature; other surgical series suggest that pregnant women represent about 1% of all subjects undergoing parathyroidectomy.[2] Some cases may be overlooked because symptoms of hypercalcemia may not be distinguished from normal constitutional symptoms of pregnancy, and because the normal pregnancy-related fall in serum albumin and PTH may mask the hypercalcemia and elevated PTH level. If the ionized calcium or albumin-corrected serum calcium are elevated with a detectable PTH level, this reliably indicates primary hyperparathyroidism during pregnancy.

The available data from case reports suggest an alarming rate of adverse outcomes for the fetus and neonate, including a 30% rate of spontaneous abortion or stillbirth. Complications have been found in up to 67% of mothers and 80% of their babies.[2] These outcomes are thought to result from fetal and neonatal hypocalcemia, which is precipitated in utero by suppression of the fetal parathyroid glands.[3] In some case reports the parathyroid suppression lasted for months after birth, and in a few it appeared to be permanent.[3] Parathyroidectomy is usually recommended during the second trimester to minimize these outcomes. Case series have reported elective surgery to be well tolerated, and to dramatically reduce the rate of adverse events when compared to earlier cases in the literature. However, those earlier cases had a severe form of primary hyperparathyroidism that is not often seen today—symptomatic, with nephrocalcinosis and renal insufficiency—and so the extent to which surgery actually reduces these outcomes in the milder cases seen today remains uncertain.

Milder, asymptomatic primary hyperparathyroidism has been followed conservatively with successful outcomes, but complications have still occurred in more cases managed medically than in those managed surgically.[71] A potential medical option is Cinacalcet, which acts on the calcium receptor to suppress PTH synthesis and release and to stimulate CT, thereby lowering the blood calcium. It is a Category C medication but has been used in pregnancy in a few cases.[72] However, the calcium receptor is expressed in placenta, fetal parathyroids, and C-cells, and so Cinacalcet may also suppress the fetal parathyroid glands, stimulate fetal CT, and alter the rate of placental calcium transfer. In the absence of definitive data, surgery during the second trimester remains the most conservative and common recommendation for primary hyperparathyroidism during pregnancy.[2,73]

3.3 Familial Hypocalciuric Hypercalcemia

This condition is caused by autosomal dominant, inactivating mutations of the calcium receptor that lead to hypercalcemia and hypocalciuria. Affected individuals develop hypercalcemia in utero and do not suffer from the long-term complications associated with primary hyperparathyroidism. Pregnancy is uneventful but maternal hypercalcemia can adversely affect the fetus and neonate. In pregnant mice bearing a mutation in the calcium receptor, the fetal parathyroids are suppressed, as indicated by a PTH level well below the fetal norm.[70] So too in humans maternal hypercalcemia has been reported to suppress the fetal parathyroids, followed by neonatal hypocalcemia and tetany, even in neonates destined to be hypercalcemic due to inheritance of the mutation.[74,75] The neonate of a woman known to have familial hypocalciuric hypercalcemia should have careful

surveillance for hypocalcemia, whereas unexpected neonatal hypocalcemia should prompt evaluation of the mother for hypercalcemia.

3.4 Hypoparathyroidism

Animal models of hypoparathyroidism during pregnancy have included thyroparathyroidectomized rats and *Pth* null mice. As noted earlier, rats typically develop secondary hyperparathyroidism in the last several days of pregnancy while the fetus is rapidly accreting calcium. Thyroparathyroidectomized rats are even more hypocalcemic while their fetuses have enlarged parathyroids, increased bone resorption, and reduced length and mineral content of the femurs.[2] But as noted earlier, intestinal calcium absorption still doubles during pregnancy in thyroparathyroidectomized rats, and this may offset the development of maternal hypocalcemia. More recently, studies in *Pth* null mice have shown that normal pregnancy-related increases in calcitriol and whole body and spine bone mineral content are achieved.[21] The *Pth* null mice bear normal litters on a typical 1% calcium diet. These findings suggest that loss of PTH may not be as disruptive to calcium homeostasis as it is in the nonpregnant rodent. However, in both thyroparathyroidectomized rats and *Pth* null mice, sudden deaths from presumed hypocalcemia can occur during the last few days of pregnancy, especially in association with anesthesia.

Among reported clinical cases and others that have been shared with the author (many detailed in Ref. 2), some hypoparathyroid women have fewer hypocalcemic symptoms and require significantly less supplemental calcium or calcitriol during pregnancy. This is consistent with the normal suppression of PTH, the rising levels of PTHrP and other hormones that affect calcium metabolism, and animal data which indicate that calcitriol and intestinal calcium absorption both increase despite complete absence of PTH. One recent case report carefully documented how a hypoparathyroid woman required progressively less calcitriol and supplemental calcium as pregnancy progressed, and was off calcitriol altogether by 28 weeks of gestation.[76] Within hours of delivery she developed severe hypocalcemia and required IV calcium and reinstitution of calcitriol supplementation until lactation became fully engaged, which implies that placental factors PTHrP contributed to her reduced need for supplemental calcium or calcitriol.[76]

It is clear from other case reports that some pregnant hypoparathyroid women require increased calcitriol supplementation in order to avoid worsening hypocalcemia. In some of these cases it is evident that the normal dilutional fall in total serum calcium was misinterpreted as worsening hypocalcemia, and led to changes in treatment despite no escalation of symptoms. In still other cases the women clearly had worsening of hypocalcemic symptoms or a demonstrable fall in ionized or albumin-corrected calcium.[2] Consequently, in some hypoparathyroid women the fetal calcium demand overwhelms a woman's ability to maintain her own blood calcium, similar to what has been seen in parathyroidectomized rats.

The ionized calcium or albumin-corrected serum calcium should be maintained in the low-normal to normal range in pregnant women because hypoparathyroidism has been associated with the development of intrauterine fetal hyperparathyroidism and death. Late in pregnancy, the doses of calcitriol and supplemental calcium may need to be reduced or eliminated in order to avoid maternal hypercalcemia caused by rising PTHrP levels.

3.5 Pseudohypoparathyroidism

There are no animal data pertaining to pseudohypoparathyroidism in pregnancy. The available human data are conflicting and echo the data on hypopararhyroidism.[2] In two cases pregnancy normalized the serum calcium level, reduced the PTH level by half, and increased the calcitriol level two- to threefold.[77] But another seven reports of pregnancy in pseudohypoparathyroid women have suggested that increased doses of calcium and calcitriol are needed to maintain the serum calcium in the normal range.[78–81] In some but not all cases there was biochemical confirmation of worsening hypocalcemia during pregnancy.

Pseudohypoparathyroidism may improve during pregnancy due to stimulatory effects of placental lactogen, PRL, and E_2 on the 1α-hydroxylase, as calcitriol levels more than doubled (similar to normal pregnancy) during the second and third trimester for two pseudohypoparathyroid women.[77] Placental production of calcitriol was normal in four pregnancies of pseudohypoparathyroid women[82] but, as noted earlier, it appears that the human placenta contributes little or no calcitriol to the maternal circulation. Normocalcemia must be maintained in the mother to reduced the risk of fetal secondary hyperparathyroidism and its neonatal consequences.[83]

3.6 Pseudohyperparathyroidism

Pseudohyperparathyroidism is PTHrP-mediated hypercalcemia, which can occur during pregnancy as a consequence of excessive release of the hormone from breasts, placenta, or both. Regardless of the source, high calcium, low PTH, and high PTHrP are the expected findings in the pregnant woman. When the breasts are the source of PTHrP, they may be normal sized or large, but the hypercalcemia persists until a bilateral mastectomy is carried out or the production of PTHrP declines postpartum (and especially after weaning).[84–89] When the

placenta is the source, the short half-life of PTHrP in the circulation results in reversal of the biochemical abnormalities within a few hours after delivery. This was illustrated by a woman with marked hypercalcemia, high PTHrP (21 pmol/L), and undetectable PTH, who within hours of an urgent C-section became profoundly hypocalcemic with undetectable PTHrP and elevated PTH.[90]

3.7 Vitamin D Deficiency and Genetic Vitamin D Resistance Syndromes

The actions of calcitriol on the vitamin D receptor are crucial for normal adult mineral homeostasis. In the available animal models, vitamin D deficiency, absence of the vitamin D receptor, or 1α-hydroxylase deficiency each result in hypocalcemia, hypophosphatemia, rickets, and sudden death due to presumed hypocalcemia. However, during pregnancy the critical role of vitamin D/calcitriol appears to be minimized.[2]

The animal models which have been studied during pregnancy include severely vitamin D-deficient rats,[91–94] pigs with a null mutation of the 1α-hydroxylase,[30] *Cyp2b1* null mice,[23] and *Vdr* null mice.[22] Vitamin D deficiency or the genetic mutations result in females that conceive less often, are hypocalcemic, hypophosphatemic, and rachitic, and bear smaller litters than normal. In each model sudden, sporadic maternal deaths may occur during late pregnancy, especially in association with anesthesia. These presumed hypocalcemic deaths may indicate that the mother has difficulty maintaining normocalcemia during the peak interval of placental–fetal calcium transfer. As noted earlier, vitamin D–deficient rats and *Vdr* null mice also upregulate intestinal calcium absorption to the same high rate achieved during pregnancy by normal rats and mice, and gain skeletal mineral content during pregnancy.[2,22] *Cyp2b1* null mice also gain significant bone mineral content while pregnant.[23] A high calcium diet increases the likelihood of conception and larger litters in *Vdr* null mice, but uneventful pregnancies still occur on a normal calcium diet.[95] Another chapter of this book details how the fetus appears relatively unaffected by maternal vitamin D deficiency, lack of 1α-hydroxylase, and absence of the vitamin D receptor. Overall these findings predict that babies born of women who are severely vitamin D deficient will likely have normal cord blood calcium and phosphorus, and normal skeletal morphology and mineral content at birth.

Multiple observational studies and clinical trials have demonstrated that maternal 25-hydroxyvitamin D levels do not change significantly during pregnancy,[2] even in women who start with the extremely low mean value of 20 nmol/L (8 ng/mL).[96] Consequently, it does not appear that pregnant women require higher intakes of vitamin D to maintain a set 25-hydroxyvitamin D level, or that the fetus represents a significant drain on maternal vitamin D stores. No large randomized trials have examined the effects of vitamin D deficiency or insufficiency on human pregnancy. However, available data from small clinical trials of vitamin D supplementation, observational studies, and case reports suggest that, consistent with animal studies, vitamin D deficiency is not associated with any worsening of maternal calcium homeostasis, and the fetus will have a normal serum calcium and fully mineralized skeleton at term (this topic is reviewed in Chapter 20 and in more detail in Refs. 2,3). The only consistent benefit seen in randomized trials is that vitamin D supplementation increases maternal and cord blood 25-hydroxyvitamin D levels without altering cord blood calcium or anthropometric parameters in the baby. High dose vitamin D has been proposed to have nonskeletal benefits, such as reduced preterm delivery, preeclampsia, vaginal infections, etc., but randomized trials using up to the equivalent of 7000 IU of vitamin D daily have found no obstetrical, fetal, or neonatal benefit apart from higher 25-hydroxyvitamin D levels.[2,3,97–103] These negative results do not preclude that vitamin D supplementation might have benefits beyond preventing rickets; however, no benefit has been demonstrated thus far.

In women with absent 1α-hydroxylase (vitamin D-dependent rickets type 1, VDDR-I) treatment with calcium and physiological doses of calcitriol or 1α-cholecalciferol should be adjusted as needed (similar to the management of hypoparathyroidism) to maintain a normal ionized or albumin-corrected serum calcium. In women with absent vitamin D receptors (VDDR-II) there may no responsiveness to calcitriol or cholecalciferol, but adjustments to the calcium content of the diet may be sufficient to maintain a normal ionized or albumin-corrected serum calcium.

3.8 Calcitonin Deficiency

Early animal models of calcitonin deficiency involved thyroparathyroidectomy followed by parathyroid gland autotransplantation and treatment with exogenous thyroid hormone.[104–108] A small (5%–10%) and borderline significant reduction in the ash weight and calcium content of goat metacarpals and metatarsal, and rat femora, were seen when thyroidectomized and sham-operated animals were compared at the end of pregnancy.[104,105,108] A more recent model used *Ctcgrp* null mice, which represent true CT deficiency because the gene encoding CT and CT gene-related peptide-α has been ablated.[109] There were no differences in *Ctcgrp* nulls compared to WT sisters in biochemistries, PTH, calcitriol, or BMC of the whole body, lumbar spine, or hindlimb, suggesting that loss of CT does not impair mineral or skeletal metabolism during pregnancy.[19,20] The surgical models provided modest evidence that an intact thyroid gland may protect the maternal skeleton from loss of bone mineral

during pregnancy, but *Ctcgrp* null mice did not reveal any such protective effect.

Increased serum CT has been proposed to protect the human skeleton from excessive resorption during pregnancy. Totally thyroidectomized women are not truly CT deficient due to production by placenta and breasts, and no women with genetic loss of CT or its receptor have been identified or studied during pregnancy.

3.9 Low Calcium Intake

Studies described earlier in rats have shown that low calcium intake will worsen secondary hyperparathyroidism in the mother during late pregnancy and cause fetal skeletal resorption.[2] This suggests that low calcium intake should be avoided during human pregnancy.

The doubling of intestinal calcium absorption and consequent absorptive hypercalciuria in most pregnant women implies that calcium intake typically exceeds maternal requirements. Intuitively a low maternal calcium intake would be harmful to mother and fetus, and low calcium intake has been found in some pregnant women who present with vertebral fractures.[69] However, clinical trials have shown mixed and inconclusive evidence of maternal or neonatal benefit from calcium supplementation during pregnancy. In one study supplemental calcium failed to alter maternal or neonatal bone density,[110] whereas a randomized trial of 2 g of calcium supplementation versus placebo found that neonatal BMD was improved only in offspring of mothers who started out in the lowest quintile of calcium intake (<600 mg/day).[111] These findings suggest that calcium supplementation may only benefit women (and their babies) who would otherwise have the very lowest intakes of calcium.

Low calcium intake has been associated with increased risk of preeclampsia. Calcium supplementation reduces the risk of preeclampsia when the dietary calcium intake is low, but there is no effect when dietary calcium intake is adequate.[112]

3.10 Hypercalcemia of Malignancy

There are no animal data pertaining to hypercalcemia of malignancy during pregnancy, but there are about ten published clinical cases and the author is aware of others.[2] Surprisingly the baby's outcome is not mentioned in most reports. The neonate may be hypercalcemic at birth but hypocalcemic with respiratory distress afterward, and one baby died out of four cases where the outcome was reported. Intravenous pamidronate has been used to control maternal hypercalcemia during pregnancy with no adverse effect noted in the neonates, and in several cases the mother died within a few months postpartum.

Treatment of hypercalcemia of malignancy during pregnancy may include debulking surgery, hydration, furosemide, and CT. Bisphosphonates and denosumab cross the placenta, and high doses may cause fetal hypocalcemia and disrupt endochondral bone development. Left untreated, maternal hypercalcemia causes fetal hypoparathyroidism with subsequent neonatal tetany. Thus the use of bisphosphonates and denosumab may be considered but only after carefully considering relative benefits and risks in each case.

3.11 FGF23-Related Disorders

Pregnancies are unremarkable in *Phex (Hyp)*$^{+/-}$ mice, a model of X-linked hypophosphatemia (XLH) that leads to FGF23 excess.[42,43,113] Despite the maternal hypophosphatemia, the fetuses display normal serum mineral and calciotropic hormone concentrations, lengths, placental phosphorus transport, skeletal development, and mineralization.[42] Hyperphosphatemic disorders due to loss of FGF23 cannot be studied during pregnancy because *Fgf23* null mice die by several weeks of age,[114] while *Klotho* null mice have marked hypogonadism and die by 9–10 weeks of age.[115]

Hypophosphatemia persists during pregnancy in women with XLH,[116,117] but it remains uncertain whether the hypophosphatemia needs to be treated. Calcitriol and phosphorus supplementation is generally maintained throughout pregnancy in order to ensure adequate delivery of phosphorus to the fetus.[118] There are no published reports on pregnancies in women with hyperphosphatemic disorders caused by deficiency of FGF23 or its coreceptor.

4 SKELETAL AND MINERAL PHYSIOLOGY DURING LACTATION

The neonatal demand for calcium begins at birth and requires an average daily transfer of 210 mg calcium into human breast milk, with much higher outputs documented in wet nurses and other women.[2] Women nursing twins or triplets respectively have double and triple the output of calcium into milk.[2] Analysis of ash weight and mineral content of human babies suggests that the neonatal skeleton accretes about 100 mg of calcium daily.[2,3]

Lactating women temporarily resorb bone to provide much of the calcium content of milk. Lactating rodents have a proportionately higher calcium demand due to their large litters (8–12 pups) and shorter duration of lactation (21 days), and they meet the demand through the combination of increased intestinal calcium absorption and resorption of 20%–30% of the skeleton.

This skeletal demineralization is accomplished by osteoclast-mediated bone resorption and osteocytic osteolysis. It is not controlled by PTH or calcitriol but

appears to be stimulated by mammary gland-derived PTHrP and permitted by low systemic E_2 levels, among other possible factors. It reverses postweaning through mechanisms that remain to be elucidated.[2]

4.1 Mineral Ions and Calciotropic Hormones (Fig. 21.3)

Early in the postpartum period the dilutional effects of pregnancy recede as the vascular volume becomes contracted; the serum albumin returns to normal. Consequently, the total serum calcium may be a reliable measurement during lactation, although the ionized calcium and albumin-corrected serum calcium are still preferred.[2]

Data from lactating rats have shown variable results for ionized or total serum calcium, with modest hypocalcemia, normal values, or even modest hypercalcemia being reported.[2] A lower maternal serum calcium can be provoked in rats or mice by dietary calcium restriction or through intensive lactation imposed by greater litter sizes. Longitudinal studies in lactating mice have found that the ionized calcium and total serum calcium are normal or even slightly elevated when nursing 6–10 pups.[12,20,22,62,63] The lactating rodent's physiology is adapted to deliver substantial calcium to the milk each day from skeletal and intestinal sources, and if that milk is not produced then rebound hypercalcemia will result. This has been observed in lactating *Ctcgrp* null mice which have excessive bone resorption during lactation and become hypercalcemic in the first 24 h after delivery before the pups are fully suckling,[20] and in normal lactating rats and mice which become hypercalcemic upon forced weaning.[2,62,119]

In longitudinal studies done in lactating women, the ionized and albumin-corrected serum increase slightly but remain normal.[2]

In lactating mice and humans, serum phosphorus levels are higher than nonpregnant values and may exceed the normal range.[2] This results from enhanced skeletal

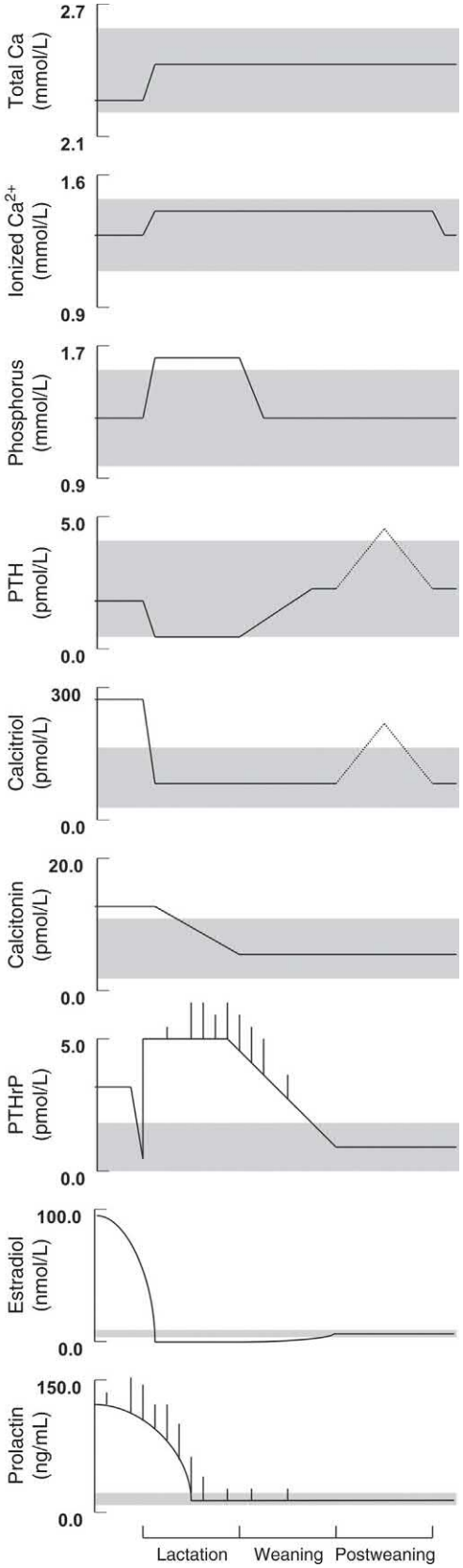


FIGURE 21.3 Schematic depiction of longitudinal changes in calcium, phosphorus, and calciotropic hormone levels during lactation and postweaning skeletal recovery in women. Normal adult values are indicated by the shaded areas. PTH does not decline in women with low calcium or high phytate intakes, and may even rise above normal. Calcidiol (25OHD) values are not depicted; most longitudinal studies indicate that the levels are unchanged by lactation, but may vary due to seasonal variation in sunlight exposure and changes in vitamin D intake. PTH-related protein (PTHrP) and prolactin (PRL) surge with each suckling episode, and this is represented by upward spikes. FGF23 values cannot be plotted due to lack of data. Very limited data suggest that calcitriol and PTH may increase during postweaning, and the lines are dashed to reflect the uncertainty. *Source: Reproduced with permission from Kovacs CS. Maternal mineral and bone metabolism during pregnancy, lactation, and post-weaning recovery.* Physiol Rev *2016;***96***(2):449–547.*[2]

release of phosphate in addition to phosphate absorbed through the diet. Urine phosphorus levels increase above the normal range,[12] likely due to the increased filtered load and phosphaturic influence of PTHrP.

Older literature suggested that PTH is elevated during lactation but relied upon outdated assays that measured biologically inactive fragments. Using modern "intact" PTH assays, lactating rats have high PTH levels which rise even higher with larger litter sizes or dietary calcium restriction.[2] In contrast, Black Swiss and C57BL/6 strains of lactating mice have suppressed PTH beginning early in lactation that remains low until weaning,[12,20,22] while PTH levels are low during the first several days but normal during the rest of lactation in CD1 mice.[62] Studies in parathyroidectomized rats and *Pth* null mice agree that PTH is not required for lactation to proceed normally, as determined by the calcium content of milk, growth of pups, or extent of bone mineral content lost during lactation.[2,21,120] In humans the intact PTH level remains low during the first several months of lactation before rising to normal or above at weaning.[2] Observational studies of hypoparathyroid women (discussed later) confirm that PTH is not required during lactation.

Calcitriol levels are two to five times normal during pregnancy and this increase is sustained during lactation in rats, whereas the values fall to normal in mice.[2,12,121] More intense lactation or dietary calcium restriction leads to higher calcitriol levels in rats, and parathyroidectomy reduces the circulating calcitriol level by 60%. These findings suggest that the increase in calcitriol is dependent in part upon secondary hyperparathyroidism in lactating rats. However, rodents do not require calcitriol for lactation to proceed normally because severely vitamin D–deficient rats, *Cyp27b1* null mice, and *Vdr* null mice lose a normal amount of skeletal mineral content during lactation.[22,23,93,122] In women free and bound calcitriol levels fall to normal within days of parturition and remain there throughout lactation; nursing of twins does not consistently affect calcitriol concentrations.[2]

CT levels are elevated during pregnancy in rodents but rapidly decline to normal. CT and its receptor are expressed in lactating mammary tissue where its role is not established, but loss of CT is associated with upregulation of PTHrP expression in mammary tissue.[2] CT is also expressed in pituitary and there is evidence that it inhibits the expression of PRL.[20,123,124]

In women, CT levels are elevated during pregnancy and remain that way for the first 6 weeks postpartum before declining to normal. CT is expressed in the lactating breast, such that in thyroidectomized women plasma CT rose from undetectable to ~14 pg/mL, while milk CT levels were over 45 times higher than these values.[32] Consequently, thyroidectomized women are not CT deficient when breastfeeding.

Plasma PTHrP levels are increased in lactating rodents and may achieve the highest values measurable apart from during late fetal development.[2,125] The source of circulating PTHrP is the lactating mammary tissue which substantially upregulates PTHrP mRNA during lactation. Suckling induces PTHrP (mRNA and protein) locally in rat mammary glands and this response appears to be mediated by PRL and blocked by bromocriptine.[2] Confirmation that the mammary glands are the source of circulating PTHrP comes from a mouse model in which the PTHrP gene was ablated within mammary tissue at the onset of lactation, and such mice have lower blood levels of PTHrP than in wild-type lactating controls.[121] Additional studies in mice indicate that PTHrP regulates the water and calcium content of milk.[2,126,127] Furthermore, as discussed later, mammary-specific deletion of PTHrP confirms that it is a key factor promoting skeletal resorption during lactation.[121]

In lactating women plasma PTHrP levels at 2–6 pmol/L are significantly higher than in nonpregnant controls, and equivalent (in molar equivalent units) to circulating PTH levels in nonpregnant adults.[2] As is true for rodents, the source of PTHrP is the breasts which effectively become accessory parathyroid-like glands during lactation. PTHrP mRNA is upregulated in mammary tissue and breast milk concentrations of the protein are 10,000 times higher than in the blood of patients with hypercalcemia of malignancy or normal controls. Suckling likely forces some PTHrP into the maternal circulation while most goes into milk; a small rise in the systemic level of PTHrP can be demonstrated after suckling. PTHrP plays a central role during lactation by stimulating resorption of calcium from the maternal skeleton, renal tubular reabsorption of calcium, and (indirectly) suppression of PTH. Clinical studies have determined that PTHrP levels correlate with the amount of BMD lost, negatively with PTH levels, and positively with the ionized calcium levels of lactating women.[2] Occasionally hypercalcemia occurs during lactation and elevated circulating PTHrP levels have been documented in some cases; the hypercalcemia resolves with weaning.[84,128] Furthermore, observations in hypoparathyroid women (discussed later) provide additional evidence of the key role that PTHrP plays in regulating maternal calcium homeostasis during lactation.

PRL is persistently elevated during early lactation and achieves its highest levels after suckling. As the postpartum days pass, basal PRL levels return to normal in between feeds but continue to spike with each suckling episode. PRL plays a key role in initiating and maintaining milk production; dopaminergic agents inhibit PRL and stop milk production. PRL and PRL receptor null mice are infertile but heterozygous-deleted PRL receptor mice demonstrate impaired lactation.[129] PRL may alter bone metabolism by stimulating PTHrP

production in lactating mammary tissue, inhibiting gonadotropin-releasing hormone (GnRH) and ovarian function, and through direct actions in osteoblasts which express the PRL receptor. Clarifying the role of PRL in regulating bone metabolism is particularly challenging because suppressing PRL inhibit milks production and mammary production of PTHrP, and disinhibits GnRH, without requiring PRL to have a direct effect on bone.

OT's known role during lactation is to contract myoepithelial cells within mammary tissue, thereby causing suckling-induced milk ejection. If not ejected, milk stasis leads to apoptosis of mammary cells and lactation ceases. OT reaches peak circulating levels in women within 10 min of the onset of suckling.[130] OT may in part regulate the differentiation and function of osteoblasts and osteoclasts, but because OT null mice cannot lactate, a role for OT in lactational bone metabolism has not been established.[131]

E_2 controls bone metabolism by inhibiting RANKL and stimulating osteoprotegerin production by osteoblasts. E_2 levels are near the detection limit in both nonpregnant and pregnant mice and are slightly but not significantly lower during lactation.[63] Estrus cycles and ovulation continue unabated during lactation in mice with simultaneous pregnancies occurring, and so it is unclear that E_2 levels are sufficiently lower in lactating mice to stimulate bone resorption. In contrast, E_2 levels reach menopausal levels in lactating women and estrogen-deficiency symptoms, such as hot flashes are common. The low E_2 levels in lactating women do stimulate bone resorption, as discussed later.

Lactation induces changes in myriad other hormones, such as luteinizing and follicle stimulating hormone, progesterone (PROG), testosterone, inhibins, and activins. Whether these play roles in regulating skeletal metabolism during lactation has not been investigated.

4.2 Calcium Pumping and Secretion in Mammary Tissue

Mammary epithelial cells pump calcium against a steep concentration gradient. It appears that calcium enters mammary epithelial cells through stretch activated and other channels, and associates with intracellular binding proteins (casein, phosphate, citrate, calbindins, etc.) and the Golgi apparatus (Fig. 21.4). About 30% of calcium transport into milk involves extrusion from the Golgi apparatus through transepithelial secretion.[132,133] The other 70% of calcium entry into milk results from plasma membrane calcium ATPase isoform 2 (PMCA2) pumping calcium across apical membranes directly into milk.[134,135]

The mammary tissue is not a simple drain through which calcium exits the mother; rather, the calcium and fluid content of milk are tightly regulated by suckling, the calcium receptor, PTHrP, PRL, and other factors.[121,126,136] Calcium regulatory genes expressed by lactating mammary tissue include the calcium receptor, PTHrP, CT receptor, 1α-hydroxylase, and vitamin D receptor.[126,137,138]

Recent studies in knockout mice have enabled some aspects of the regulation of calcium transport into mammary epithelial cells and secretion into milk to be understood. PTHrP plays a key role because loss of mammary-derived PTHrP at the onset of lactation leads to reduced bone turnover markers, less bone resorbed, and lower milk calcium content.[121] Conversely, the expression of PTHrP in mammary tissue and the circulation is increased by low E_2, low dietary calcium, high PRL, loss of CT, suckling, and treatment with a bisphosphonate to reduce bone resorption.[2,19,20,63,126,139] The central controller of calcium transport in mammary epithelial cells is the locally expressed calcium receptor which regulates the fluid and calcium content of milk, and mammary production of PTHrP[2,126,127] (Fig. 21.5). A calcimimetic blocks the effect of a low calcium diet to increase mammary production of PTHrP,[126] while ablation of the calcium receptor upregulates mammary gland PTHrP and reduces calcium transport into milk.[127] The calcium receptor stimulates calcium secretion into milk by upregulating expression of PMCA2, expressed on the apical plasma membranes of mammary epithelial cells.[126,134] A null mutation in *Pmca2* leads to milk that has low calcium content.[134] In this way the lactating mammary gland uses the calcium receptor to sense availability of calcium (Fig. 21.5). It stimulates calcium secretion into milk when the supply is adequate, and stimulates the production of PTHrP when the calcium supply is waning. PTHrP's main role is the delivery of calcium to mammary epithelial cells, while PMCA2 controls milk calcium content.

Mice lacking CT and CT gene-related peptide-α display upregulation of PTHrP within mammary tissue and a doubling of milk calcium content, although it remains unknown whether these are direct effects of loss of CT expression within mammary tissue.[19,20]

The demands of the breasts for calcium can provoke maternal hypocalcemia, as observed in rats and mice nursing large litters, and in the condition known as milk fever in cows.

4.3 Intestinal Absorption of Calcium

Lactating rodents maintain a twofold higher rate of duodenal calcium absorption, similar to pregnancy.[2] This may not be dependent upon calcitriol as severely vitamin D–deficient rats and, by inference, *Vdr* null mice have upregulated intestinal calcium absorption during lactation.[51,52,140] Other factors, such as PRL may stimulate intestinal calcium absorption. Upregulated intestinal

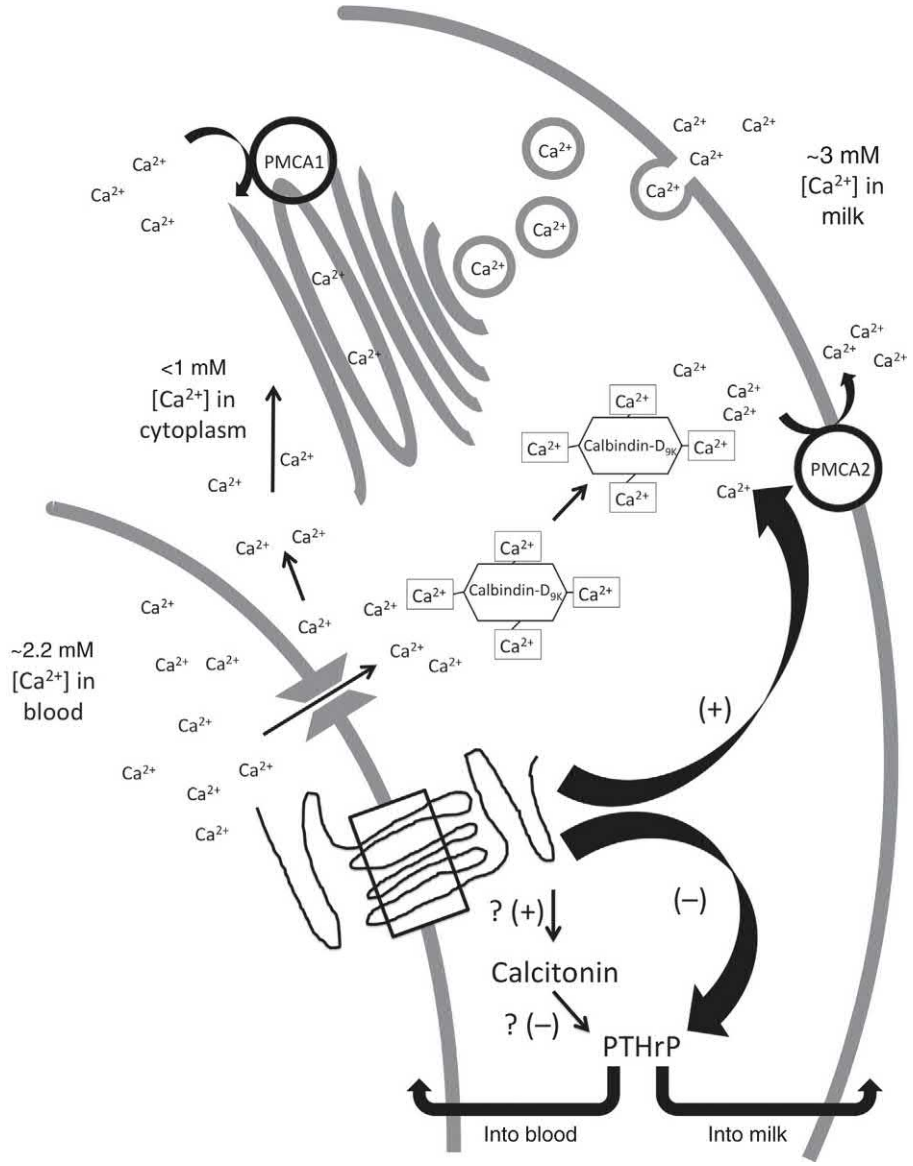


FIGURE 21.4 Calcium transport across mammary epithelial cells into milk. The basolateral (left) and apical (right) basement membranes of a mammary epithelial cell are depicted. Calcium enters the cell through channels that have not been defined; transient receptor potential vanilloid (TRPV6 and TRPV5) are not expressed. About 20%–30% of calcium destined for secretion into milk is pumped into the Golgi apparatus via plasma membrane calcium ATPase (PMCA1), wherein it is packaged into secretory granules containing proteins (casein and α-lactalbumin) and complexes of calcium with phosphate and citrate. These granules are extruded from the Golgi apparatus into milk through transepithelial secretion at the apical membrane. About 70%–80% of calcium entering the cell becomes bound to carrier proteins, such as calbindin-D9k and is shuttled to the apical membrane in the transcellular pathway, from where PMCA2 actively pumps the calcium into milk. Calcium ions binding to the calcium receptor on the basolateral membrane inhibit PTHrP and stimulate PMCA2 to pump calcium into milk. Calcitonin (CT) may also influence these processes, perhaps through regulation by the calcium receptor (which regulates CT in C-cells of the thyroid), as absence of CT causes upregulation of PTHrP within mammary epithelial cells and milk calcium concentration is also increased. PTHrP is released into milk at higher concentrations than it is released into the maternal circulation.

calcium absorption is important in rodents as a low calcium diet causes hypocalcemia and reduced calcium content of the milk.[2,126]

In lactating humans, intestinal calcium absorption promptly decreases to the nonpregnant rate from the doubled rate of pregnancy.[2] Therefore, although rodents require upregulation of intestinal calcium and skeletal resorption to meet the calcium demands of lactation, humans rely mainly on skeletal resorption of mineral.

4.4 Renal Handling of Calcium

Lactating rats and mice show decreased renal calcium excretion.[2,12,20] Similarly, in lactating women the glomerular filtration rate falls during lactation, and renal excretion of

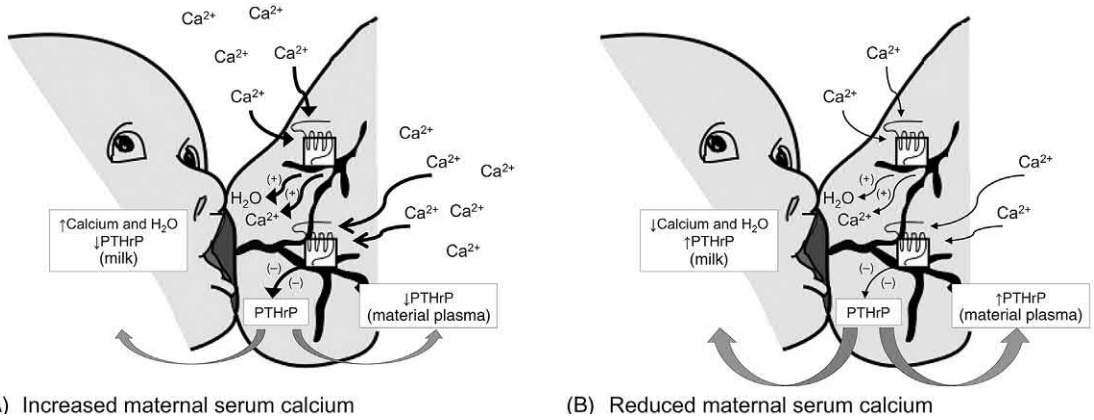


FIGURE 21.5 The role of PTHrP and calcium receptor (CaSR) within the lactating breast. The calcium receptor (represented schematically) is expressed by lactating mammary epithelial cells. It monitors the systemic concentration of calcium to control PTHrP synthesis and, thereby, the supply of calcium to the breast. An increase in serum calcium or administration of a calcimimetic inhibits PTHrP expression (A), whereas a decrease in serum calcium or ablation of the calcium receptor from mammary epithelial cells stimulates PTHrP expression (B). The calcium receptor also directly regulates the calcium and fluid composition of milk independent of PTHrP. Administration of a calcimimetic stimulates calcium and water transport into the breast, while ablation of the calcium receptor results in low milk calcium despite increased PTHrP and systemic hypercalcemia. PTHrP produced by mammary epithelial cells enters the maternal circulation to stimulate maternal bone resorption and renal calcium conservation. It also enters milk at 1,000–10,000-fold higher concentrations, from where it may influence neonatal accrual of calcium.

calcium is typically reduced to levels as low as 50 mg/24 h.[2] These findings suggest that tubular reabsorption of calcium must be increased, perhaps through the actions of PTHrP. This renal calcium conservation contrasts with pregnancy where urinary calcium excretion increases and may exceed the normal range.

4.5 Skeletal Calcium Metabolism

Marked skeletal resorption occurs during lactation in all mammals that have been studied, including rodents and nonhuman primates. Bone formation and resorption markers are both elevated with the most marked increase occurring in resorption markers. Histomorphometric data from lactating animals show increased bone formation but a greater increase in bone resorption parameters.[2] Through a process called osteocytic osteolysis, osteocytes resorb their surrounding matrix to provide additional mineral during lactation.[2,141] This can be visualized by the increased size and prominence of the lacunae that surround osteocytes. The combined effects of osteoclast-mediated resorption and osteocytic osteolysis (Fig. 21.6) cause a net loss of bone mass and mineral content, with more marked reductions at trabecular than cortical sites, and the greatest losses at the lumbar spine. The decline in mineral content has been assessed by determining the total or trabecular ash weight of bone, the mineral content of the ash, and by serial measurement of bone mineral content or density with DXA. By these methods the trabecular mineral content may decline 20%–35% during 3 weeks of normal lactation in the rat and mouse. Greater losses (40% or more) will occur in rodents on a calcium-restricted diet and in response to larger litter sizes, and these changes are sufficient to decrease the mechanical properties of bone and predispose to fracture.[2] Conversely, a calcium-rich diet can suppress the loss of bone during lactation. A 2% calcium intake reduced the skeletal losses incurred by lactation in mice while a 1.4% calcium intake did not alter bone loss in rats.[21,142]

Lactating women also show evidence of increased bone turnover with net resorption of bone, although the extent of loss is much less than in rodents.[2] No histomorphometric data are available. Biochemical markers of bone formation and resorption have been assessed

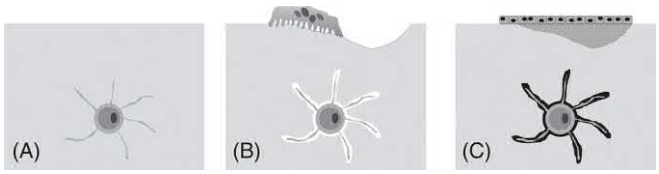


FIGURE 21.6 Osteocytic osteolysis and osteoclast-mediated bone resorption. (A) Quiescent bone with an osteocyte surrounded by its lacuna and canaliculi (*gray* halo and tentacles). (B) Lactation with an osteoclast resorbing bone (resorption pit) while an osteocyte resorbs mineral from its lacuna and pericanalicular spaces (surrounding white regions). (C) Postweaning phase, during which osteoblasts restore bone in areas previously resorbed by osteoclasts (hatched resorption pit), and osteocytes remineralize their lacuna and pericanalicular spaces (surrounding black regions). Evidence from osteocyte-specific ablation of the PTH/PTHrP receptor in mice suggests that bone resorption and osteocytic osteolysis may each account for about 50% of the mineral lost from the skeleton during lactation. *Source: Reprinted with kind permission from Kovacs CS, Ralston SH. Presentation and management of osteoporosis presenting in association with pregnancy or lactation.* Osteoporos Int *2015;***26***(9):2223–41, copyright 2015 Springer Science and Business Media B.V.*[69]

in numerous cross-sectional and longitudinal studies. Potential confounding must be considered as the glomerular filtration rate is reduced during lactation and the intravascular volume is contracted. In general, bone formation and resorption markers are elevated over prepregnancy and pregnancy values, or those obtained in nonpregnant controls, and the most marked elevation occurs in bone resorption markers. Loss of the placenta leads to a rapid fall in total alkaline phosphatase but it may remain above normal due to elevation in the bone-specific fraction.

Changes in bone mineral content have been assessed by serial measurements with SPA, DPA, or DXA. The results are quite variable between individual women and between studies with a decline of 3%–10% in bone mineral content being observed after 2–6 months of lactation at trabecular sites (particularly lumbar spine), with smaller losses at cortical sites and whole body.[2,67] The median loss from the lumbar spine is approximately 8% at 6 months based on all available studies.[2] Some studies in lactating adolescents demonstrated losses of 10%–15% from baseline.[2] The skeletal losses occur at rates of 1%–3% *per month*; by comparison, a loss of more than 1%–2% *per year* is considered rapid in postmenopausal women. Although rodents may resorb less bone on a very high-calcium diet, several interventional studies have demonstrated that calcium supplementation does not reduce the amount of bone lost by women during lactation.[143–146] In a randomized clinical intervention trial that studied the effect of consuming 2.4-g calcium daily, lactating women still lost 6.3% of BMD at the lumbar spine and up to 8% from the radius and ulna, as determined by DXA.[146] The lactational decrease in BMD correlates with the breast milk output,[147] and predicts that women who nurse twins will lose more bone mineral content than women who nurse singletons.

The mechanism for the loss of bone mass and mineral content during lactation has been investigated in several animal models. While not fully explained, low E_2 and increased PTHrP are key factors driving the bone resorption. Estrogen replacement given to mice at the start of lactation reduced the extent of bone lost, but the achieved E_2 levels were nearly eight times the normal value for lactation, and about four times the virgin level.[125] Consequently, those high doses overestimate the contribution of estrogen deficiency in causing bone loss during lactation. Other studies suggested that estrogen deficiency plays only a small role. Ovariectomy in rodents leads to lower E_2 levels than during lactation but the rate of bone loss is slow, typically being assessed several months after surgery as compared to 3 weeks for lactation.[2,125] Also, ovariectomized rats and simultaneously pregnant and lactating rats each lose the same amount of bone as intact rats going through normal lactation.[2] E_2 deficiency induced with GnRH analogues causes little or no loss of skeletal mineral content in several strains of mice as compared to normally lactating controls[125] (and unpublished data).

PTHrP plays a significant role in stimulating bone resorption during lactation was confirmed by the previously described conditional knockout of PTHrP from mammary tissue. Those mice had reduced serum calcium, calcitriol, nephrogenous cAMP, bone turnover markers, and net loss of bone mineral content.[121]

The Wysolmerski lab attempted to replicate the skeletal effects of lactation in virgin mice by continuously administering PTHrP together with E_2 deficiency induced either by a GnRH analogue or by ovariectomy. No significant bone loss occurred from the combined interventions, and the authors concluded that factors other than PTHrP and E_2 deficiency must contribute to lactational bone loss.[125] However, the study was confounded by lack of the pumping drain (i.e., the lactating breast) which actively disposes of resorbed skeletal mineral during lactation. The serum calcium was higher in the combination treatment group as compared to normal lactation, and PTH was suppressed. The higher serum calcium and lack of a "drain" allowed calcium to be recycled back into bone and likely suppressed bone resorption.

PTH is not required as shown by normal lactational losses occurring in parathyroidectomized rats and *Pth* null mice.[2,21] Similarly severely vitamin D–deficient rats and *Vdr* null mice also experience normal lactational losses of mineral content.[22,93,122] As noted earlier, loss of CT more than doubled skeletal losses in mice, which confirms that CT normally protects the maternal skeleton against excessive losses during lactation, at least in rodents.[20] The mice showed not only structural loss of trabecular bone but also prominence of osteocytic lacunae, an indicator of increased osteocytic osteolysis.[148]

Human data also indicate that estrogen deficiency is unlikely to be the main explanation for the speed and magnitude of skeletal resorption that occurs during lactation. Ovariectomy or normal menopause leads to an average annual loss of 1% of skeletal mineral content. Six months of acute estrogen deficiency induced in young women by GnRH agonist therapy leads to 1%–4% losses in trabecular (but not cortical) aBMD, increased urinary calcium excretion, and suppression of calcitriol and PTH.[2] However, during lactation, women are not as estrogen deficient and lose more aBMD (at both trabecular and cortical sites), have normal (as opposed to low) calcitriol levels, and have reduced (as opposed to increased) urinary calcium excretion. The difference between isolated estrogen deficiency and lactation is likely due to the effects of PTHrP which synergizes with estrogen deficiency to markedly stimulate skeletal resorption (Fig. 21.7). Additional data indicate that PTHrP likely causes the bone loss during lactation. In one study of lactating women, higher PTHrP levels correlated with loss of BMD at the

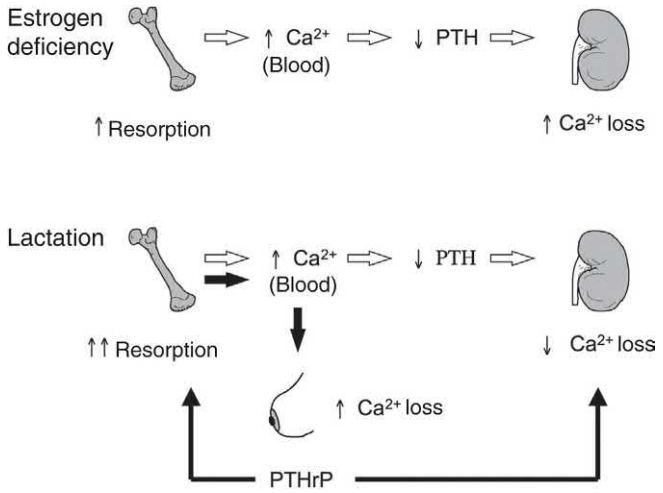


FIGURE 21.7 Schematic depiction of the effects of acute, isolated estradiol (E_2) deficiency from gonadotropin-releasing hormone (GnRH) analog therapy versus lactation. Acute estrogen deficiency (e.g., GnRH analog therapy) increases skeletal resorption and raises the blood calcium; in turn, PTH is suppressed and renal calcium losses are increased. During lactation, the combined effects of PTHrP (secreted by the breast) and estrogen deficiency increase skeletal resorption, reduce renal calcium losses, and raise the blood calcium, but calcium is directed into breast milk. *Source: Reprinted from Kovacs CS, Kronenberg HM. Maternal–fetal calcium and bone metabolism during pregnancy, puerperium, and lactation.* Endocr Rev *1997;***18***(6):832–72, copyright 1997 the Endocrine Society.*[1]

lumbar spine and femoral neck, even after accounting for the effects of E_2 levels, PTH, and breastfeeding status.[149] Case reports mentioned earlier have demonstrated lactational hypercalcemia associated with high PTHrP and increased bone resorption, with postweaning resolution of the hypercalcemia and high PTHrP.

In animal models the decline in mineral content is fully restored after lactation. Histological studies have shown rapid apoptosis of osteoclasts within 24 h of weaning while osteoblast numbers increase further from the high values that were present during lactation.[62,148,150] An interval of rapid bone formation leads to reversal of thinned trabecular plates and cortices, and the depleted mineral content that developed during lactation.[20,62] Osteocytes stimulate bone formation in their lacunae, as demonstrated by the appearance of tetracycline labels postweaning and a return of lacunar size to normal[151] (Fig. 21.6). Bone biomarkers indicate a marked suppression of bone resorption while the bone formation markers maintain the high levels achieved during lactation or increase further.[12,62] The striking difference between lactation and weaning is the sudden and marked suppression of osteoclast numbers and bone resorption parameters at weaning, which leads to uncoupled bone formation by surging numbers of osteoblasts. By DXA, the spine, hindlimb, and whole body return bone mineral content to normal or above within 14 days for Black Swiss mice, and by 21–28 days in other strains of mice.[20,62] By microCT, the bone mass returns to normal at differing rates by each skeletal site, with some taking much longer time than others.[152] The factors that stimulate bone formation during the postweaning interval remain unknown. Studies in parathyroidectomized or severely vitamin D-deficient rats, and genetically engineered mice, have shown that PTH, PTHrP, CT, vitamin D, and the vitamin D receptor are not required, and that deficits as great as 55% of skeletal mineral content, as observed in CT-deleted mice, are fully recovered.[2,12,20–22]

In humans, the bone density losses during lactation appear to be substantially reversed during weaning,[2,67,144] although the speed and completeness of recovery may differ by skeletal site and technique used. This corresponds to a gain in bone density of 0.5%–2% per month in a woman who has weaned her infant. As with the animal models, the mechanism that regulates this interval of marked bone formation is uncertain. Restoration of menses often precedes the start of skeletal recovery and indicates that E_2 levels have likely normalized; however, it does not prove that E_2 stimulates postweaning skeletal recovery. Resumption of menses also indicates that lactation is now less intense, skeletal resorption can decline, and this may simply facilitate skeletal recovery. Clinical experience with estrogen replacement in postmenopausal women has shown that it suppresses osteoclasts but is not a major stimulator of bone formation, and so E_2 is unlikely to account for skeletal recovery after lactation. Moreover in the clinical studies of women who had E_2 deficiency induced for 6 months by treatment with GnRH analogues and lost only 1%–4% of bone mineral content, most of that small deficit persisted a year after return of normal ovarian function.[2]

These short-term losses in bone mass and mineral content cause at most a transient increase in skeletal fragility but no long-term effect in most women. There have been dozens of studies analyzing risk factors for fracture among pre- and postmenopausal women. The vast majority of these studies have found no adverse effect of a history of lactation or lifetime duration of lactation on peak bone mass, bone density, or hip fracture risk; indeed, some studies found that lactation confers a protective effect against future risk of fracture.[2] So too in adolescents it appears that lactation does not imperil the achievement of peak bone mass as previously feared. In an NHANES III analysis of 819 women aged 20–25, women who breastfed as adolescents had higher BMD than women who had not breastfed and nulliparous women.[68] Thus it appears that lactating adolescents and adults recover fully without any long-term adverse effects on the skeleton.

4.6 Brain–Breast–Bone Circuit

The foregoing data suggest that there are key interactions between brain, breast, and bone during lactation (Fig. 21.8). In response to suckling, the pituitary produces

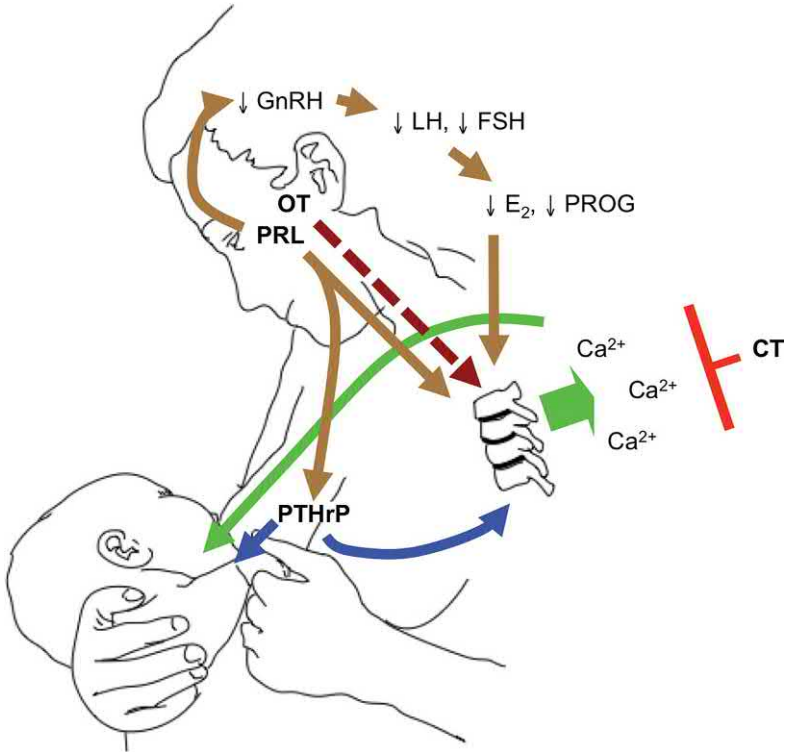


FIGURE 21.8 Breast–brain–bone circuit controls lactation. Suckling and PRL both inhibit the hypothalamic GnRH pulse center, which in turn suppresses the gonadotropins [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)], leading to low levels of the ovarian sex steroids [eE_2 and progesterone (PROG)]. Prolactin may also have direct effects on its receptor in bone cells. PTHrP production and release from the breast are stimulated by suckling, prolactin, low E_2, and the calcium receptor. PTHrP enters the bloodstream and combines with systemically low E_2 levels to markedly upregulate bone resorption and (at least in rodents) osteocytic osteolysis. Increased bone resorption releases calcium and phosphate into the blood stream, which then reaches the breast ducts and is actively pumped into the breast milk. PTHrP also passes into milk at high concentrations, but whether swallowed PTHrP plays a role in regulating calcium physiology of the neonate is uncertain. In addition to stimulating milk ejection, oxytocin (OT) may directly affect osteoblast and osteoclast function (*dashed line*). CT may inhibit skeletal responsiveness to PTHrP and low E_2 given that mice lacking CT lose twice the amount of bone during lactation as normal mice. Not depicted is that CT may also act on the pituitary to suppress prolactin release, and within breast tissue to reduce PTHrP expression and lower the milk calcium content (see text). *Source: Adapted with kind permission from Kovacs CS. Calcium and bone metabolism during pregnancy and lactation.* J Mammary Gland Biol Neoplasia *2005;***10***(2):105–18, copyright 2005 Springer Science and Business Media B.V.*[153]

PRL. Suckling-induced nerve pathways and PRL inhibit gonadotropin releasing hormone in the hypothalamus, in turn suppressing the pituitary gonadotropins [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] and, thereby, ovarian function. The resulting low E_2 levels upregulate RANKL and downregulate OPG in osteoblasts, thereby stimulating the differentiation and function of osteoclasts and promoting bone resorption. Suckling, PRL, and low E_2 also stimulate PTHrP production by mammary tissue, and suckling forces the release of PTHrP into the maternal circulation from where it synergizes with low E_2 levels to stimulate osteoclast-mediated bone resorption and osteocytic osteolysis. CT is produced by mammary tissue where it may have local actions to regulate the calcium content of milk or PTHrP production, and systemic actions (together with thyroid-derived CT) to suppress osteoclast-mediated bone resorption and inhibit PRL. OT may act not only on the breast to stimulate milk ejection but also on bone to control the differentiation and function of osteoblasts and osteoclasts.

5 DISORDERS OF BONE AND MINERAL METABOLISM DURING LACTATION

5.1 Osteoporosis of Lactation

Lactation disrupts the microarchitecture of bone and reduces its material properties, particularly within the lumbar spine. However, rodents are not susceptible to vertebral compression fractures, likely because ambulation on all fours reduces loading of the spine. No spontaneous fractures were noted in *Ctcgrp* null mice despite a 55% decrease in the mineral content of the spine during lactation.

Ambulating on the hindlimbs loads the axial skeleton; consequently, humans are susceptible to vertebral crush fractures when the material properties of the spine are compromised. Yet for the vast majority of women the

lactational declines in bone density are silent; only rarely do women suffer fragility fractures.[69] In such cases an osteoporotic bone density may be confirmed, but low bone mass prior to pregnancy generally cannot be excluded due to lack of prior DXA measurements. Secondary causes of osteoporosis may be present, including anorexia nervosa, hyperparathyroidism, osteogenesis imperfecta, and corticosteroid or heparin therapy.[69] Some cases might occur when otherwise healthy women with low bone mass cannot tolerate the normal magnitude of skeletal losses during lactation, whereas others may result from excessive bone resorption during lactation, such as from overabundant production of PTHrP by mammary tissue. In at least one case of lactational osteoporosis, PTHrP levels were high for months after weaning.[154] If a woman is known to have severe osteoporosis or very low bone density prior to pregnancy, it may be reasonable to discourage breastfeeding on the presumption that additional skeletal losses would compromise skeletal integrity and lead to fractures.

For most women with confirmed fractures, pharmacological treatment may be unnecessary due to spontaneous increases in bone mass postweaning and typically self-limited duration of any pain.[2,69] A case series of women who presented with vertebral compression fractures during lactation found that bone density increases spontaneously after weaning, consistent with the evidence that bone loss during lactation preceded the fracture but is reversible.[155] Various case reports have described the anecdotal and uncontrolled use of bisphosphonates, strontium, or teriparatide for lactational osteoporosis, but whether the achieved increment in bone density exceeds what occurs naturally postweaning remains unknown.[2,69] Treatment might be best reserved for the most severe cases. Most women can be reassured that the problem does not usually recur after subsequent pregnancies.

5.2 Primary Hyperparathyroidism

There are no animal studies of the effects of primary hyperparathyroidism during lactation, nor are there any human data. However, as lactation programs marked bone resorption due in part to the combined effects of PTHrP and low E_2, hypercalcemia may worsen with breastfeeding. A decision to breast feed may influence whether a parathyroidectomy should be done postpartum.

5.3 Familial Hypocalciuric Hypercalcemia

Mice heterozygous for an inactivating mutation of the calcium receptor lactate normally and their pups grow at the expected rate, but PTHrP expression is upregulated above normal in mammary tissue, while the milk has higher PTHrP and lower calcium content.[127]

There have been no human studies during lactation but the animal data predict that women with familial hypocalciuric hypercalcemia may have more marked PTHrP-stimulated skeletal resorption but a lower calcium content of milk.

5.4 Hypoparathyroidism

Studies of parathyroidectomized rats and *Pth* null mice have shown that lactation proceeds normally, although the mice are prone to sudden postanesthetic deaths from presumed hypocalcemia. In both models bone mass increases after weaning, indicating that bone recovery does not require PTH.[2,21]

Observational studies in women confirm that PTH is not required for normal lactation.[2] There may be increased risk of hypocalcemia in the first day or two, after loss of placental PTHrP and before the milk has come in. Once lactation is established the requirement for supplemental calcitriol and calcium falls substantially, and significant hypercalcemia has occurred when these adjustments haven't been made.[156] In one case high circulating PTHrP levels coincided with a rise in calcitriol and serum calcium.[157] A single case report found a spontaneous 40% increase in lumbar spine BMD and 7.5% increase in femoral neck BMD in a surgically hypoparathyroid woman after weaning, confirming the animal data that substantial skeletal recovery can occur despite the absence of PTH.[158]

5.5 Pseudohypoparathyroidism

No animal models of pseudohypoparathryoidism have been studied during lactation, and management in lactating women has not been documented. The requirement for supplemental calcitriol might well increase during lactation if low E_2 aggravates the renal resistance to PTH. On the other hand, these patients do not have skeletal resistance to PTH and so excessive resorption from the skeleton may occur when high PTHrP and low E_2 levels are added to the mix during lactation. As the outcome is uncertain, these women should be observed more closely with adjustments in calcium and calcitriol intake made as needed.

5.6 Pseudohyperparathyroidism

Occasionally breastfeeding leads to symptomatic, PTHrP-mediated hypercalcemia.[88,154,159,160] It has also developed during the puerperium in a woman who was unable to breastfeed because her baby was critically ill,[84] and in non-lactating women with large breasts.[85–87,161] High levels of PTHrP cause excess skeletal resorption, while intestinal calcium absorption may also increase if production of calcitriol is sufficiently stimulated.

Weaning, breast binders, and dopaminergic medications, should each help reverse the excess production of PTHrP and consequent hypercalcemia. A bisphosphonate or denosumab may also be needed to shut down bone resorption, especially as seen in some women wherein the excess production of PTHrP has persisted for months after weaning.[2]

5.7 Vitamin D Deficiency and Genetic Vitamin D Resistance Syndromes

The animal data described earlier demonstrated that the skeleton resorbs during lactation under the influence of PTHrP, low E_2, and possibly other hormones. Vitamin D and calcitriol do not appear to play a significant role as severely vitamin D–deficient rats and *Vdr* null mice lactate normally and have a normal magnitude of bone loss. After weaning, bone formation increases and *Vdr* null mice restore the lost bone mass, but rats differ on the extent of bone restoration postweaning.[22,93,122]

Very little vitamin D or 25-hydroxyvitamin D passes into milk; consequently, women's 25-hydroxyvitamin D levels are usually unchanged by lactation.[162,163] Case reports, observational studies, and small clinical trials indicate that lactation proceeds normally regardless of vitamin D status, and that breast milk calcium content is unaffected by vitamin D deficiency or by supplementation with doses as high as 6400 IU per day (topic reviewed in detail in Ref. 2). No studies have examined the impact of vitamin D deficiency or insufficiency on skeletal recovery after weaning; however, as dozens of epidemiological studies found no adverse effect of number and duration of lactation on the risk of low bone mass or osteoporosis, and vitamin D insufficiency is common among reproductive age women, it seems probable that skeletal recovery after weaning does not require vitamin D.

5.8 Calcitonin Deficiency

CT deficiency during lactation cause *Ctcgrp* null mice to lose twice the normal amount of bone mineral content during lactation but they fully restore it after weaning.[19,20] The milk calcium content is doubled but whether this is the result of increased availability calcium from bone resorption, or altered calcium transport in the mammary tissue, remains unclear.[19,20] These findings indicate that physiological levels of CT protect the rodent skeleton from excessive resorption during lactation, but it may reflect actions of CT not only in osteoclasts, but also in pituitary lactotrophs and mammary epithelial cells.[20]

Whether CT deficiency alters the physiology of human lactation is unknown. Studies have not been done in lactating thyroidectomized women, and might not be definitive anyway because extrathyroidal production of CT causes a significant increase in circulating CT during lactation. A recent case report described a thyroidectomized woman nursing twins who experienced multiple vertebral compression fractures during lactation; the authors speculated that CT deficiency contributed to marked bone loss confirmed by DXA.[158]

5.9 Low Calcium Intake

Rodents depend upon multiple sources of calcium during lactation, including increased intestinal calcium absorption, decreased renal calcium excretion, and enhanced demineralization of the skeleton. A low calcium intake accentuates skeletal losses and increases the risk of sudden death from presumed hypocalcemia.[2] These findings indicate that, while not optimal, the rodent can derive most of the required calcium from the skeleton. There are no data on the sufficiency of calcium intake needed to ensure skeletal recovery after lactation.

In humans the calcium content of milk may be largely derived from skeletal resorption. Low calcium intake does not alter breast milk calcium content nor does it accentuate maternal bone loss during lactation.[164–167] Conversely high calcium intake also fails to alter breast milk calcium or skeletal resorption during lactation.[143–146] These findings suggest that it may not matter what calcium intake a women has during lactation, but it is remains prudent to maintain the recommended dietary intake of calcium. No studies have examined calcium requirements during the postweaning interval.

5.10 FGF23-Related Disorders

Phex$^{+/-}$ females have excess FGF23 and remain hypophosphatemic and normocalcemic during lactation, with no adverse effects noted on their pups.[113] Milk from *Phex*$^{+/-}$ females has normal phosphorus, calcium, and protein content.[113] Hyperphosphatemic disorders due to loss of FGF23 cannot be studied due to early mortality of *Fgf23* null and *Klotho* null mice.

Serum phosphorus normalized during lactation in a woman with XLH,[116] likely the result of the normal increase in skeletal resorption. However, milk phosphorus content was 50% of normal in two cases,[116,117] and normalized with oral phosphorus supplementation.[116] Low milk phosphorus likely contributed to hypophosphatemia observed in the babies.

6 CONCLUSIONS

During pregnancy and lactation, novel regulatory systems are invoked to meet the challenges for increased calcium and phosphorus delivery. The fetal calcium demand is met by a doubling of intestinal calcium absorption beginning early in pregnancy, an adaptation that

may not be fully explained by a doubling or tripling in calcitriol levels. There may be some contribution of calcium from skeletal stores whereas the maternal kidneys output more calcium than normal during pregnancy. During lactation, skeletal resorption is the dominant mechanism by which calcium is supplied to the breast milk. Renal calcium conservation occurs and only minimal dietary intake of calcium may required. These lactational changes appear to be driven by PTHrP (facilitated by the estrogen deficiency) and not by PTH, calcitriol, or FGF23. Consistent with this, women with very low or high intakes of calcium experience a similar degree of bone loss during lactation. The rapidity of calcium regain by the skeleton of the lactating woman occurs through a mechanism that is not understood. Finally, while a few women will experience fragility fractures in association with pregnancy or lactation, for the vast majority of women these adaptations in calcium and bone metabolism during pregnancy and lactation are silent, without long-term adverse consequences.

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SECTION 3

DISORDERS OF BONE AND JOINT

CHAPTER

22

Osteoporosis Genes Identified by Genome-Wide Association Studies

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1 INTRODUCTION

Without doubt the field of osteoporosis (just as most of all other medical fields) was revolutionized by the advent of the Human Genome Project[1] at the end of the 1980s. An exponential increase in the number of publications in the field was observed shortly after its launch with the trend continuing to its completion in the year 2001, and even more so thereafter (Fig. 22.1). This impressive wave of studies concerning the genetics of osteoporosis in humans has now begun a new era of discoveries with the advent of so-called genome-wide association studies (GWAS),[2–4] whose design has been comprehensively described in Chapter 3. Before the GWAS era, the literature about the genetics of osteoporosis and fracture had been confined to a very large number of "genome-wide linkage" and "candidate gene association" studies. With few exceptions, the majority were small, inadequately-powered studies generating controversial and frequently nonreproducible reports[5] on variants in about 150 candidate gene regions for osteoporosis (HUgeNet website: https://www.cdc.gov/genomics/hugenet/hugenavigator.htm/). Some shortcomings were tackled in the GENOMOS consortium where large-scaled evidence (n = 20,000–45,000 which, by current standards, is still substantial) was produced on a limited number of "the usual suspects" in genetics of osteoporosis including the estrogen receptor type 1 (*ESR1*),[6] *VDR*,[7] *COLIA1*,[8] *TGFB1*,[9] and low density lipoprotein receptor-related protein 5 (*LRP5*)/*6*[10] genes. These results are summarized in Table 22.1. Although a few polymorphisms were indeed identified as being associated with either bone mineral density (BMD) or fracture (such as for *LRP5* very significantly associated at $p < 5 \times 10^{-8}$, the current standard for declaring genome-wide significance), this effort was restricted to known polymorphisms and did not interrogate the genetic contribution to osteoporosis at a genome-wide level as is now possible in GWAS.

For most complex traits and common diseases[11–13] hundreds if not thousands of variants of weak (but real) effects[14] will be underlying the genetic architecture of the trait and disease. From this perspective, only well-powered studies based on several independent populations (for replication), with a well-defined selection of polymorphisms and gene regions, and a robust control for multiple hypothesis-testing in the analysis, will be suited to identify genuine genetic effects.[15] While the GWAS approach (with some few exceptions) incorporates in its design several of these properties, the number of loci identified depends on the total sample size obtained in a given study: more samples will lead to more GWAS "hits." Therefore, to the present, only a small fraction of all the variants expected to be associated with a complex trait have been detected so far. In addition, some of these loci discovered at a genome-wide significant level [(GWSL) i.e., 1 in 20 or $P = 0.05$ after the Bonferroni correction] may still represent false-positive reports. Nevertheless, after replication in tens to hundreds of thousands of individuals, it is very likely that genome-wide significant signals associated at a stringent $P < 5 \times 10^{-8}$ will be associated with variants which represent true underlying biological mechanisms influencing the trait in question.

Another important aspect for the interpretation of the GWAS discoveries is that, with few exceptions, most of the genes claimed as "underlying the GWAS signals" have been labeled as such without robust functional evidence supporting or demonstrating its candidacy. Usually, allocating a gene to a genetic signal is based on physical distance and current knowledge of biology, neither of which suffices to assign unequivocally the gene to the single nucleotide polymorphism (SNP) effect. Therefore, even though the circumstantial evidence for biologic candidacy may be strong, such assignments should be interpreted with caution until conclusive

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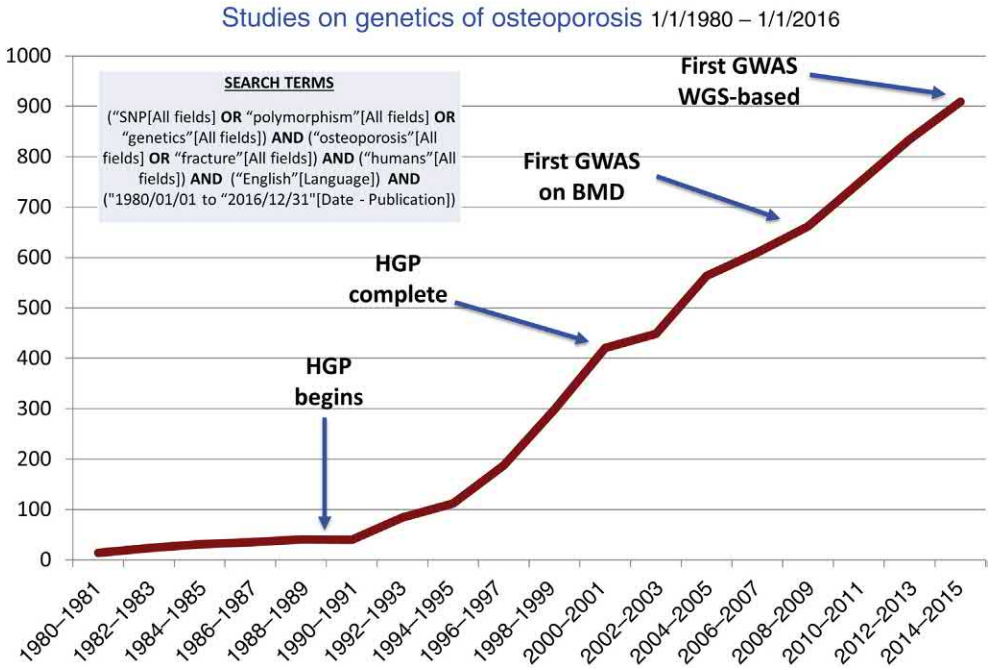

FIGURE 22.1 **Historical trend of the number of publications in the field of "genetics of osteoporosis.** BMD, Bone mineral density; GWAS, genome-wide association studies; WGS, whole-genome sequencing.

TABLE 22.1 Pre-GWAS Results of the GENOMOS Consortium

			BMD		Fracture		
Gene	**SNPs (*n*)**	**Sample (*n*)**	**Femoral neck (SD)**	**Lumbar spine (SD)**	**Vertebral (%)**	**Nonvertebral (%)**	**References**
ESR1	3	18,917	—	—	20–30	10–20	6
VDR	5	26,242	—	—	10	—	7
COL1A1	1	20,786	0.15	0.15	10	—	8
TGFB1	5	28,924	—	—	—	—	9
LRP5	2	37,760	0.15	0.15	12–26	6–14	10
LRP6	1	37,760	—	—	—	—	10

BMD, Bone mineral density; *ESR1*, estrogen receptor type 1; GWAS, genome-wide association studies; *LRP5*, low density lipoprotein receptor-related protein 5, *LRP6*, low density lipoprotein receptor-related protein 6.

evidence becomes available. This will also vary from region to region depending on the relative location of the associated SNPs, the linkage disequilibrium (LD) properties between the markers, the patterns of recombination rate, and the number of genes underlying the signal. All of these parameters must be considered while evaluating the candidacy of the gene(s) underlying the GWAS signals. From this perspective, the likelihood of a gene being responsible for a GWAS signal will be a function of the properties of the signal and the candidacy characteristics (known biological function) of the gene (Table 22.2).

Nevertheless, just as for any candidate gene evaluation, these assessments will be based on previous knowledge about the gene, which by itself constitutes a liability considering that the GWAS approach is a hypothesis-free screen of the genome. This indicates that new and not (properly) annotated areas of the genome will be found in a given GWAS, leaving much new biology to be discovered. This way, not scoring high on the candidacy dimension may just reflect the lack of knowledge, and does not exclude any gene from being truly involved in the biologic mechanisms leading to trait variation.

In addition, the follow-up of these GWAS discoveries requires robust functional evidence linking the human genome sequence to the function of its regulatory elements. This is being facilitated by the Encyclopedia of DNA Elements (ENCODE) Project, which has started a revolution by going beyond the "linear" configuration of regulatory elements challenging the previous context of boundaries and gaps between genes.[16] The knowledge derived from Encyclopedia of DNA Elements provides

TABLE 22.2 Likelihood of a Gene Being Responsible for a GWAS Signal

A. Properties of the GWAS signal arising from a SNP (or a variant in high LD)

1. location in relation to the gene (exonic, intronic, promoter, 3'UTR or intergenic),
2. functionality (promoter/regulatory region, nonsynonymous, synonymous, transcription factor binding, or splice regulation),
3. physical distance from the gene, and
4. colocation in the same LD block of the gene.

B. Candidacy characteristics by which a gene is weighted based on the presence of

1. SNP (or a variant in high LD) constituting an eQTL associated with gene expression,
2. KO model organism (mouse or zebra fish) with a skeletal phenotype,[a]
3. monogenic human syndrome (OMIM) with a skeletal phenotype[a] and
4. involvement in a bone-active pathway.[a]

eQTL, Expression quantitative trait locus; LD, linkage disequilibrium; SNP, single nucleotide polymorphism.
[a] *Candidacy in relation to osteoporosis traits.*

new approximations to the understanding of diverse processes of gene regulation including chromatin interactions, epigenetic states, promoter activities, enhancer binding, and nuclear lamina occupancy to name a few. This knowledge will provide large leaps in the identification of the gene(s) underlying the GWAS signals and in the understanding of new biology.

2 GENOME-WIDE ASSOCIATION STUDIES OF OSTEOPOROSIS

We define the set of candidate osteoporosis genes included in this chapter as those proposed to have been discovered by the GWAS approach within adequately powered settings (n > 10,000 subjects) in relation to BMD, the most accessible and up to now the most prolific of the osteoporosis traits. An overview of GWAS studies on osteoporosis is presented in Table 22.3. The first GWAS report concerning osteoporosis was published in 2007 by Kiel et al.[17] The effort failed to identify any associated loci at a GWSL as a reflection of the limited sample size (n = 1141) and sparse SNP content (100 K) of the study. In 2008, in Richards et al.,[18] variants in *LRP5* and *TNFRSF11B* (OPG) were reported as associated with lumbar spine and femoral neck BMD in 8557 United Kingdom and Dutch individuals. Almost simultaneously, Styrkarsdottir et al. published a report on 5861 Icelandic individuals, with replication in an additional 7925 European individuals,[19] which identified variants mapping also to *TNFRSF11B* (OPG), together with additional ones mapping to the *TNFSF11* (RANKL), *ESR1*, *ZBTB40*, and the major histocompatibility complex loci. A subsequent report from this group published in early 2009 was based on an extended set including 6865 Icelandic individuals, with replication in another 8510 European

TABLE 22.3 GWAS of BMD

GWAS populations	Discovery	Total	Reported loci	References
Framingham osteoporosis study	1,141	1,141	0	17
Twins UK and the Rotterdam study	2,094	8,557	2	18
deCODE 1	5,861	13,786	5	19
ALSPAC	1,518	5,474	1	21
deCODE 2	6,865	15,375	4	20
KARE	8,842	16,703	2	22
GEFOS 1	19,125	19,125	20	23
Hong Kong osteoporosis study	800	18,898	1	24
AOGC	1,955	20,898	2	25
GEFOS 2	32,961	83,894	56	26
GEFOS-SEQ (UK10K)	2,882 WGS + 32,961		56 + 2 rare	27
UKBIOBANK	142,487		203	28

WGS, Whole-genome sequencing.

individuals.[20] They identified variants mapping to *TNFRSF11A* (RANK), *SOST*, *MARK3*, and *SP7* (osterix). Shortly afterward, variants in the osterix gene were also identified by Timpson et al. in an effort based on 1518 United Kingdom children, followed by replication in adults, including an "extremes truncate selection" of 132 Australian individuals with high or low BMD, and in 3692 individuals of European descent.[21] During mid-2009, Cho et al. published a study examining ultrasound of the radius, tibia and, the heel in 8842 Korean individuals (with replication in additional 7861 individuals) postulating *FAM3C* and *SFRP4* as new BMD loci.[22] Though speed of sound ultrasound does not directly measure BMD, it is associated with fracture risk. At the end of 2009, the genetic factors of osteoporosis (GEFOS) consortium reported a large leap in the discoveries described in Rivadeneira et al.[23] Within the setting of the GEFOS consortium (http://www.gefos.org/), variants were identified in 13 additional loci in a study of 19,195 individuals of Northern European origin. These 13 loci reached genome-wide significance for the first time and included *WLS* (former *GPR177*), *SPTBN1*, *CTNNB1* (β-catenin), *MEPE/SPP1/IBSP*, *MEF2C*, *STARD3NL*, split hand and foot malformation 1 (*SHFM1*), *LRP4*, *SOX6*, *DCDC5*, *FOXC2*, *CRGR1*, and *HDAC5*. After that, two different studies of BMD, using extreme-ascertainment designs based on 800 Chinese and on 1,955 Australian individuals, provided evidence for additional loci after replication in nearly 20,000 individuals (18,898 and 20,898, respectively). In the first study, Kung et al.[24] identified variants in JAG1 associated at a GWSL of $p < 5 \times 10^{-8}$. In the second study, Duncan et al.[25] identified variants mapping to *GALNT3* and *RSPO3* associated at a GWSL, while variants in *SOX4*, *LTBP3*, and *CLNC7* were suggestive of association. With the exception of variants that mapped to major histocompatibility complex and *LBT3*, all other loci described in this chapter have been found associated at GWSL by the recently published second effort on BMD of the GEFOS consortium,[26] which brought the number of identified BMD loci to 56.

To date, about 70 loci have been reported in the literature to be associated with BMD across different populations and age groups (Fig. 22.2). After the successful identification of common variants, most of the attention is now placed on the identification of less frequent and rare variants, which are proposed to be enriched with functional allelic variants.[29] From this perspective it is expected that variants of less-common and rare frequencies can exhibit stronger effect sizes than common variants. Recent efforts in the field of osteoporosis employing whole-genome sequencing (WGS) followed by imputation to larger populations, have proved successful identifying less frequent to rare variants and constitute relative pioneer work in the field of complex traits.[27,30–32] Through the application of WGS of Icelandic individuals, a mutation (C376T) within the *leucine rich repeat-containing G-protein-coupled receptor 4* (LGR4) gene was identified as associated with low BMD and increased risk of fractures.[30] A recent GWAS study drawn in 20,162 Icelandic individuals, and with replication in 10,091 subjects from two studies of European background and other two of East Asian descent,[32] identified variants mapping to a new locus harboring the *PTCH1* gene, the receptor for the three hedgehog morphogens (SHH, IHH, and DHH) as well as new signals across several known loci. In a subsequent effort, two rare mutations in *COL1A2* (MAF = 0.1%) were identified through WGS and shown to be associated with low BMD in 2,894 cases and 206,875 controls derived from the Icelandic population without other signs of osteogenesis imperfecta.[31] Finally, in an effort using the UK10K/1000GP sequencing reference to impute less frequent variants across 53,236 individuals from 27 population-based cohorts of European ancestry,[27] a novel less-frequent (minor allele frequency, MAF = 1.7%) noncoding variant near the *engrailed-1* (*EN1*) gene was identified, exerting large effects on BMD and fracture, with effect size about fourfold greater than those observed for any previously reported common variant.

3 GENES IDENTIFIED BY GENOME-WIDE ASSOCIATION STUDIES ON BONE MINERAL DENSITY

Table 22.4 lists 45 genes from 31 loci with variants consistently associated with BMD at a GWSL of $p < 5 \times 10^{-8,}$ including four with variants of low frequency recently identified with the use of WGS reference panels.

3.1 1p31.3 *WLS*

The (*Drosophila*) wntless homolog is a 135 kDa gene formerly known as G protein-coupled receptor 177 (*GPR177*) gene. WLS is a novel factor part of the highly evolutionary conserved Wnt signaling pathway involved in bone cell differentiation and development. The reported variants, showing association with lumbar spine and femoral neck BMD,[23] map to a narrow LD block within an intronic region of the gene. WLS has been shown to be required for cell surface expression of Wnt3a proteins by HEK cells, and capable of activating nuclear factor-kappa B (NF-κB) when expressed in HEK cells.[34] In addition, Wls has been shown to be a Wnt trafficking regulator in mouse embryogenesis.[35–37]

3.2 1p36.12 *ZBTB40* and *WNT4*

ZBTB40 is a 102 kb gene located in a well-recognized linkage region. GWAS signals in *ZBTB40* were

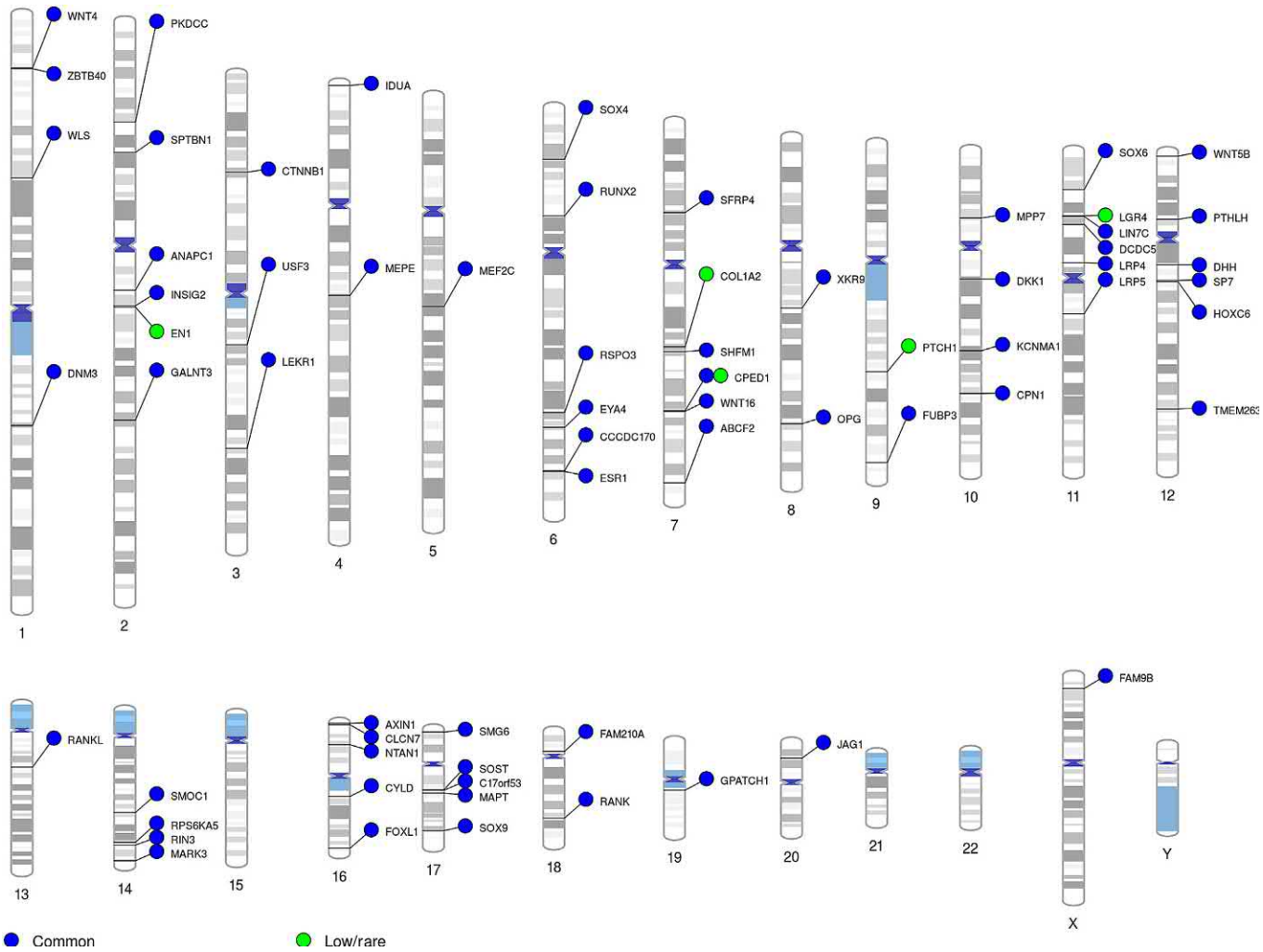

FIGURE 22.2 **Karyogram illustrating the location of identified BMD loci and candidate genes in the genome.**

first reported by Styrkarsdottir et al.[19] as associated with hip and spine BMD, and further replicated in the subsequent GWAS metaanalyses of the GEFOS consortium.[23] In the latter effort, there was evidence for an independent signal mapping to the 5′ region of *WNT4*, a smaller gene of size 27 kb located about 310 kb upstream from *ZBTB40*. *ZBTB40* encodes a protein of unknown function but which is expressed in bone. Given its zinc finger and BT domains, it is likely the protein has DNA binding properties and may be involved in protein–protein interactions. Wnt4 belongs to the Wnt family of signaling proteins and is involved in several developmental processes including regulation of cell fate and patterning during embryogenesis[38] by inhibition of β-catenin. Wnt4 is involved in the control of female development and the prevention of testes formation; mutations in the gene have shown to produce sex reversal syndromes.[39] During hematopoietic stem cell differentiation, WNT4 acts as a noncanonical activator of Wnt signaling,[40] though its role on bone remains unknown.

3.3 2p16.2 *SPTBN1*

SPTBN1 is a 215 kb gene that encodes a subform of β-spectrin, which is a major cytoskeletal scaffold protein. This locus was first reported by Styrkarsdottir et al.[20] as associated with lumbar spine BMD with subsequent replication at GWSL in the first GEFOS metaanalysis.[23] The functional role of this gene in bone remains unclear although, in mice, targeted inactivation of Elf (mouse homolog) results in disruption of transforming growth factor-beta (TGFβ) signaling with severe phenotypic alterations across multiple systems similar to that observed in double knockouts (KOs) of SMAD3 and SMAD4 proteins.[41]

3.4 2q14.2 *EN1*

EN1 is a small gene spanning 6 kb that, as for other Homeobox containing genes, exerts an important role in development. In Drosophila, the "engrailed" (en) gene plays an important role, determining segmentation during

TABLE 22.4 List of BMD Loci With Their Candidate Genes and Functional Biological Annotations

Chromosomal location	Gene name	Description	Size (kb)	MIM gene accession	MIM morbid accession	MIM morbid description	eQTL	MGI KO mouse with skeletal phenotype	Pathway member
1p31.3	WLS	Wntless homolog (Drosophila)	134.7	611514			NR_040077	MGI:1915401	Wnt
1p36.12	ZBTB40	Zinc finger and BTB domain containing 40	102.3	612106			NM_014870		
	WNT4	Wingless-type MMTV integration site family, member 4	26.7	603490			NM_030761	MGI:98957	Wnt
2p14.2	EN1	Engrailed Homeobox 1	6.0	131290				MGI:95389	
2p16.2	SPTBN1	Spectrin, beta, nonerythrocytic 1	215.2	182790					
2q24.3	GALNT3	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc-T3)	47.1	601756	211900	Tumoral calcinosis, hyperphosphatemic, familial; HFTC	NM_004482	MGI:894695	
3p22.1	CTNNB1	Catenin (cadherin-associated protein), beta 1, 88 kDa	65.3	116806			NM_001098209	MGI:88276	Wnt
4q22.1	IBSP	Integrin-binding sialoprotein	12.9	147563			NM_004967	MGI:96389	
	MEPE	Matrix extracellular phosphoglycoprotein	25.4	605912			NM_001184697	MGI:2137384	
	SPP1	Secreted phosphoprotein 1	7.8	166490				MGI:98389	Endochondral ossification
5q14.3	MEF2C	Myocyte enhancer factor 2C	187.1	600662	613443	Mental retardation, stereotypic movements, epilepsy, and/or cerebral		MGI:99458	Endochondral ossification
6p22.3	SOX4	SRY (sex determining region Y)-box 4	5.9	184430				33	
6q22.33	RSPO3	R-spondin 3	80.9	610574					Wnt
6q25.1	ESR1	Estrogen receptor 1	472.9	133430	608446	Myocardial infarction, susceptibility to, 1		MGI:1352467	
7p14.1	SFRP4	Secreted frizzled-related protein 4	119.8	606570	265900	Pyle disease		MGI:892010	Wnt
	STARD3NL	STARD3 N-terminal like	52.4	611759			NM_032016		
7q21.2	COL1A2	Collagen type I alpha 2 chain	36.7	120160	130060	Ehlers–Danlos syndrome, type VIIB		MGI:88468	
					166210	Osteogenesis imperfecta, type II			
					259420	Osteogenesis imperfecta, type III			

					166220	Osteogenesis imperfecta, type IV			
					166710	Osteoporosis, postmenopausal			
7q21.3	SHFM1	Split hand/foot malformation (ectrodactyly) type 1	228.3	601285	183600	Split-hand/foot malformation 1	NM_006304		
	C7orf76	Chromosome 7 open reading frame 76							
7q31.31	FAM3C	Family with sequence similarity 3, member C	48.1	608618					
	WNT16	Wingless-type MMTV integration site family, member 16	15.7	606267				MGI:2136018	Wnt
	CPED1	Cadherin like and PC-esterase domain containing 1	308.8				NM_001105533		
8q24.12	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b	28.6	602643	239000	Paget disease, juvenile		MGI:109587	OPG/RANK/RANKL
9q22.32	PTCH1	Patched homolog 1 (Drosophila)	74.1	601309				MGI:105373	Hedgehog
11p11.2	LRP4	Low density lipoprotein receptor-related protein 4	61.8	604270	212780	CLSS	NM_014756	MGI:2442252	Wnt
	ARHGAP1	Rho GTPase activating protein 1	23.5	602732			NM_024741	MGI:2445003	
	F2	Coagulation factor II (thrombin)	20.3	176930	613679	Prothrombin deficiency, congenital			
11p14.1	DCDC5	Doublecortin domain containing 5	539.4	612321			NM_020869		
	LIN7C	Lin-7 homolog C, crumbs cell polarity complex component	12.4	612332			NM_018362		Wnt (canonical)
	LGR4	Leucine rich repeat containing G protein-coupled receptor 4	106.8	606666	615311	Susceptibility to low BMD	NM_018362		Wnt and mTOR
11p15.1	SOX6	SRY (sex determining region Y)-box 6	773.1	607257				MGI:98368	Endochondral ossification
11q13.2	LRP5	Low density lipoprotein receptor-related protein 5	136.7	603506	607636	van Buchem disease, type 2		MGI:1278315	Wnt
					144750	Hyperostosis corticalis generalisata, benign form of worth, with torus palatinus		MGI:1278315	Wnt
					259770	OPPG		MGI:1278315	Wnt
					608084	Bone mineral quantitative trait locus 1, high bone mass included		MGI:1278315	Wnt

(*Continued*)

TABLE 22.4 List of BMD Loci With Their Candidate Genes and Functional Biological Annotations (*cont.*)

Chromosomal location	Gene name	Description	Size (kb)	MIM gene accession	MIM morbid accession	MIM morbid description	eQTL	MGI KO mouse with skeletal phenotype	Pathway member
12q13.12	DHH	Desert hedgehog	5.4	605423			NM_021044		Hedgehog
12q13.13	SP7	Sp7 transcription factor	18.7	606633	613849	Osteogenesis imperfecta, type XI	NM_001173467	MGI:2153568	
13q14.11	TNFSF11	Tumor necrosis factor (ligand) superfamily, member 11	45.3	602642	259710	OPTB2	NM_016248	MGI:1100089	OPG/RANK/RANKL
14q32.32	MARK3	MAP/microtubule affinity-regulating kinase 3	118.5	602678			NM_001128920		
16p13.3	CLCN7	Chloride channel 7	30.6	602727	611490	OPTB4		MGI:1347048	
	AXIN1	Axin 1	74.1	603816			NM_003502	MGI:1096327	Wnt
16q24.1	FOXL1	Forkhead box L1	5.8	603252					TGF-beta
	FOXC2	Forkhead box C2 (MFH-1, mesenchyme forkhead 1)	2.5	602402	153400	Lymphedema–distichiasis syndrome		MGI:1347481	Wnt
17q12-22	CRHR1	Corticotropin-releasing factor receptor 1	213.9				NR_037772		
17q21.31	SOST	Sclerostin	5.1	605740	269500	Sclerosteosis; SOST	NM_025237	MGI:1921749	Wnt
	HDAC5	Histone deacetylase 5	46.9	605315			NM_005474		
18q21.33	TNFRSF11A	Tumor necrosis factor receptor superfamily, member 11a, NFKB activator	66.0	603499	612301	OPTB7	NM_003839	MGI:1314891	OPG/RANK/RANKL
					602080	Paget disease of bone 2, early-onset			
					174810	Osteolysis, familial expansile			
20p12.2	JAG1	Jagged 1	36.3	601920	601920	Jagged 1		MGI:1095416	Notch

BMD, Bone mineral density; CLSS, Cenani–Lenz syndactyly syndrome; eQTL, expression quantitative trait locus; OPPG, osteoporosis-pseudoglioma syndrome;OPTB2, osteopetrosis, autosomal recessive 2; OPTB4, osteopetrosis, autosomal recessive 4; OPTB7, osteopetrosis, autosomal recessive 7.

development and is required for the formation of posterior compartments. While the exact role of EN1 in human bone metabolism remains to be elucidated, En1 has also been shown to interact with Wnt factors for the regulation of limb patterning in mice.[42] The identification of *EN1* influencing BMD variation and fracture risk, illustrates the advantage provided by using large sequencing-based reference panels to detect variants with low minor allele frequency, which can then be tested for association through imputation in larger GWAS collections.[27] The identified associated variant had a low frequency (MAF = 1.7%) and an unforeseen large effect size (+0.24 SD) on BMD, which is several fold greater than those typically observed with common variants (averaging ~0.02 SD). A recent GWAS in Icelandic individuals[32] identified additional less frequent variants (1.5%) in this *EN1* locus, appearing to hold stronger associations with increased BMD and lower risk of osteoporotic fracture. Further functional and fine-mapping efforts are needed to determine the genetic variants driving the strong associations in this locus.

3.5 2q24.3 *GALNT3*

GALNT3 is a gene of 47 kb encoding for an enzyme involved in biosynthesis of oligosaccharides and has high homology to other members of the GalNAc-transferases family members. Variants in and around *GALNT3* were first reported as associated at GWSL with both hip and lumbar spine BMD in Australian and Northern European populations.[25] Mutations in the GALNT3 gene cause autosomal recessive hyperostosis–hyperphosphatemia syndrome.[43]

3.6 3p22.1 *CTNNB1*

This 65 kb gene encodes β-catenin, a key transcription factor of the Wnt signaling involved in osteoblast differentiation from mesenchymal stem cells[44,45] and regulation of osteoclast activation. β-Catenin dependent (so-called canonical) Wnt signaling is a major regulator of chondrogenesis, osteoblastogenesis, and osteoclastogenesis.[46] Deletion of the gene in osteoblasts results in osteopenia while stabilization results in high bone mass.[47] Common variants located in the 5′ region of the gene were found associated for the first time at GWSL with femoral neck BMD in the first GEFOS metaanalysis.[23]

3.7 4q22.1 IBSP, MEPE, and SPP1

This locus clusters a series of relatively small phylogenetically-related genes encoding for matricellular phosphoglycoproteins important for bone formation and mineralization[33] including *MEPE*, *IBSP* (integrin binding sialoprotein), and *SPP1* [osteopontin (OPN)]. All three genes are expressed in bone and exhibit a skeletal phenotype when deleted. MEPE spans 25 kb and encodes one of these phosphoglycoproteins. Targeted inactivation of Mepe in mice results in increased BMD.[48] Even though the first GEFOS metaanalysis[23] showed that the variants achieving GWSL for association with lumbar spine BMD mapped close to *MEPE*, the involvement of the other genes in the region cannot be ignored. In a previous report, Styrkarsdottir et al.[20] reported variants mapping close to IBSP as suggestive of association with hip BMD.

IBSP is a 13 kb gene located about 42 kb away from *MEPE*, encoding a major structural protein of the bone matrix that binds tightly to hydroxyapatite and appears to form an integral part of the mineralized matrix. In addition, Koller et al.[49] showed that the effect on IBSP was also present in premenopausal women. Ibsp KO mice have impaired bone growth and mineralization, concomitant with dramatically reduced bone formation with thinner cortices, but greater trabecular bone volume than wild-type mice, and low rates of skeletal turnover,[50] indicating impairments of both new bone formation and osteoclast activity.

SPP1 is a small gene spanning 7 kb (located about 122 kb away from *MEPE*) that codes for OPN. Mice with deletion of the *OPN* gene are resistant to ovariectomy–as well as unloading–induced bone loss.[50,51]

3.8 5q14.3 *MEF2C*

MEF2C is a large 187 kb gene encoding a member of the MADS box transcription enhancer factor 2 (MEF2) family of proteins. *MEF2C* has been primarily implicated in muscle function, although it is known to play a role in endochondral ossification[51a] and is a potential regulator of the *SOST* gene by interaction with a conserved enhancer, which is deleted in van Buchem's disease.[52] The first report implicating variants in *MEF2C* as associated with BMD variation in Northern Europeans came from the first GEFOS metaanalysis[23] that has subsequently been replicated.[26] In addition, variants in *MEF2C* have also been associated with human stature.[12]

3.9 6p22.3 *SOX4*

SOX4 is a small intronless gene of about 6 kb encoding a member of the SOX (SRY-related HMG-box) family of transcription factors. *Sox4* homozygous KO mice die, but heterozygous mice display osteopenia and reduced bone strength.[53] Further, in rat osteoblasts, *Sox4* has been shown to be involved in cellular proliferation and differentiation, functioning upstream of *Osx* (Osterix) but independently of *Runx2*. Variants in and around *SOX4* presented with suggestive evidence for association with both hip and lumbar spine BMD in Australian and Northern European populations.[25] This was replicated at a GWSL in the second GEFOS metaanalysis on lumbar spine and femoral neck BMD,[26] with variants mapping in between *CDKAL1* and *SOX4*.

3.10 6q22.33 *RSPO3*

RSpo proteins are general regulators of canonical Wnt signaling, acting through a common biochemical mechanism involving competition with *DKK1* and reduction of the internalization of *LRP6* by the cell.[54] Homozygous RSPO3 KO mice display embryonic lethality, and no mutations in humans have been reported to date. Variants upstream from *RSPO3*, a gene spanning 81 kb, were first reported as associated at GWSL with BMD of the femoral neck and at nominal level with lumbar spine BMD in Australian and Northern European populations.[25] In a recent GWAS combining Icelandic, (other) European and East Asian populations,[32] a new spine BMD signal implicating *RSPO3* was identified through strong correlation with gene expression. The identified variant of moderately low frequency (6.8%) was associated with increased spine BMD levels and a protective effect on osteoporotic fracture.

3.11 6q25.1 *ESR1*

The *ESR1* gene is a very large gene, spanning 473 kb, which historically has been a strong candidate for genetic regulation of bone mass. Three main polymorphisms have been thoroughly studied in many candidate gene studies, including a TA microsatellite repeat in the promoter region, the PvuII and the XbaI restriction fragment length polymorphisms. None of these polymorphisms is associated with BMD variation, as reported by the prospective large-scale individual level metaanalysis from the GENOMOS study involving 18,917 individuals. However they did show a significant association with fracture that was independent of BMD.[55] The first GWAS report postulating variants in the *ESR1* locus as associated at GWSL with BMD variation in humans was performed in Icelandic individuals with replication on other North-Western European populations.[19] This study showed signals mapping to *C6orf97*, while this and other independent variants (not in LD) mapping within *ESR1* were subsequently replicated in larger GWAS.[20,23] Interestingly, this *ESR1* locus not only displays multiple signals (allelic heterogeneity) for BMD, but also for other phenotypes also assessed by GWAS, including breast cancer[56] risk and body height.[12]

3.12 7p14.1 *SFRP4* and *STARD3NL*

Variants in this locus were first reported associated at GWSL with BMD derived from forearm, heel, and finger ultrasound in Korean populations[22] and with lumbar spine BMD in the first GEFOS metaanalysis in European individuals[23] mapping in between the *STARD3NL* and *SFRP4* genes.

STARD3NL is a 52 kb long gene which encodes a cholesterol endosomal transporter but its potential role on bone metabolism remains unclear.

In contrast, *SFRP4* is known to inhibit Wnt signaling and, as shown in SAMP6 mice, *Sfrp4* negatively regulates bone formation and decreases BMD.[57] This inhibition of canonical Wnt signaling is suggested to be mediated by lower responsiveness for the Wnt3A ligand in osteoblasts derived from the SAMP6 strains. Attenuation of canonical Wnt signaling by overexpression of *SFRP4* in osteoblasts results in reduction of trabecular bone mass;[58] a finding consistent with lower BMD and ultrasound properties of the trabecular-rich bone content of the lumbar spine and heel skeletal sites.

3.13 7q21.2 *COL1A2*

The *COL1A2* gene encodes the pro-alpha2 chain of type I collagen whose triple helix comprises two alpha1 chains and one alpha2 chain. Type I collagen is found in most connective tissues being particularly abundant in the cornea, dermis, tendon, and bone tissues. Mutations in this gene are associated with diverse disorders of the connective tissues including osteogenesis imperfecta (types I–IV), familial osteoporosis, Ehlers–Danlos syndrome (type VIIB and the recessive form of the classical type) and atypical Marfan syndrome. As compared to the phenotype resulting from mutations in alpha1 gene (COL1A1), mutations in COL1A2 result in milder compromise across tissues. While previous large GWAS failed to identify common variants associated with osteoporosis traits in either of these genes[23,26,27], recent work using WGS data in Icelanders identified two rare (frequency 0.05%–0.10%) missense mutations, (p.Gly496Ala and p.Gly703Ser) in the *COL1A2* gene that associate with measures of osteoporosis.[31] While carriers of these mutations presented with low BMD at the hip and spine, they lack any sign of osteogenesis imperfecta (other than low BMD), implying that mutations in COL1A2 can affect skeletal phenotypes in distinct ways.

3.14 7q21.3 *SHFM1* and *C7orf76*

This locus was first described in the first GEFOS BMD metaanalysis as holding several variants associated at GWSL with both lumbar spine and femoral neck.[23] These variants map close to *C7orf76*, an open reading frame sequence lacking the classical hallmarks of protein-coding genes. The large LD block harboring the GWAS signal also includes the *SHFM1* gene, also known as "deleted in split-hand/split-foot 1" region or *DSS1* gene. More than a single gene, the region is characterized by genomic rearrangements leading to deletion of *DSS1* and the distalless-related homeogenes *DLX5* and *DLX6*. The latter two genes, code for members of the Wnt signaling pathway and cause ectrodactyly in mice and humans[59] when both are deleted or mutated. This may also be as a consequence of "functional haploinsufficiency" when

the chromosomal rearrangement physically separates the genes from their control elements. The ectrodactyly syndrome is a genetically heterogeneous limb developmental defect characterized by the absence of digital rays and syndactyly of the remaining digits.[60]

3.15 7q31.31 *FAM3C*, *CPED1*, and *WNT16*

Genetic variants in the region mapping close to *FAM3C* spanning 48 kb have been reported in association with derived BMD and speed of sound as measured by quantitative ultrasound in the radius and calcaneus in a Korean population.[22] This chromosome 7 region harbors in addition two other genes including *WNT16* (spanning 16 kb) and *CPED1* (previously known as *C7orf58* and spanning 309 kb). The success of the GWAS approach is well characterized by the discovery of this as a BMD locus, containing among others *WNT16*, now established as a critical molecule in bone biology. In the second GEFOS BMD metaanalysis, variants mapping to *WNT16* were found associated at GWSL with femoral neck and lumbar spine BMD, and also with increased fracture risk.[26] After that, several subsequent GWAS efforts identified independently the same locus when performed in premenopausal women[49]; wrist BMD[61]; total body BMD in children and adults[62]; cortical thickness from pQCT of the tibia[61]; and quantitative ultrasound of the heel[63]; postulating the important influence of *WNT16* on multiple skeletal traits and across different age groups in the life course. After being identified by GWAS, subsequent studies in murine models have started to elucidate the regulatory mechanisms of WNT16. These studies illustrated the skeletal phenotype of Wnt16 KO mice characterized by reduced cortical bone thickness, increased cortical bone porosity, and consequent increased susceptibility to spontaneous fractures.[64] This fragility specific to cortical bone is the consequence of two processes: loss of direct osteoclastogenesis inhibition via noncanonical Wnt pathway activation and loss of an indirect effect of inducing OPG expression in osteoblasts. Furthermore, subsequent work by the same investigators demonstrated how overexpression of *WNT16* increases trabecular bone mass independently of estrogen action.[65] On the other hand, it has also become clear in this locus, independent secondary signals arise from *CPED1*. Different GWAS have identified associations pointing to this gene arising from less frequent variants in the region,[27] associated with BMD at other skeletal sites (i.e., skull)[66] and/or in young populations (i.e., pediatric BMD)[62].

3.16 8q24.12 *TNFRSF11B* (OPG)

Large-scale confirmation that variants in *TNFRSF11B* are associated at GWSL with lumbar spine and femoral neck BMD were first reported in British and Dutch populations (i.e., the Twins UK and the Rotterdam Study)[18] together with other efforts involving Icelandic and Northern European populations (deCODE genetics).[19] This finding was subsequently replicated by several additional efforts[23,25,26,67,68] including Asian populations.[68] The *tumor necrosis factor receptor superfamily member 11b* is a gene that spans 29 kb, which encodes the osteoprotegerin (OPG) protein, an endogenous inhibitor of bone resorption. OPG is integral to the OPG/RANK/RANKL pathway whose relevance in the regulation of bone resorption has been well established.[69] In addition, mutations in this gene have been identified in juvenile Paget's disease (Chapter 26)[70] The functional mechanisms by which *TNFRSF11B* alleles predispose to osteoporosis are incompletely understood but, in the Twins UK and the Rotterdam GWAS, expression of the BMD lowering allele was associated with reduced expression of *TNFRSF11B* in lymphoblasts.[18]

3.17 9q22.32 *PTHC1*

The *Patched homolog 1* gene encodes a member of the patched gene family. The encoded protein is the receptor for sonic hedgehog (SHH), a secreted molecule implicated in the formation of embryonic structures and in tumorigenesis, as well as the desert hedgehog (DHH) and the Indian hedgehog proteins (IHH). Variants mapping to this locus and implicating *PTHC1* were identified very recently by a recent GWAS study drawn in individuals of Icelandic, (other) European and East Asian decent.[32] Association with BMD near the DHH gene, encoding one of the ligands for PTCH1, has previously been reported (see locus 12q13.12 later). The importance of the hedgehog-signaling pathway in skeletal development and metabolism is well established. The pathway is involved in the processes of osteoblastogenesis and chondrocyte differentiation, intramembranous ossification of cranial bones and the endochondral ossification in the rest of the skeletal system.[71] Another form of indirect regulation of osteoblasts on osteoclasts occurs via IHH signaling, inducing osteoclast maturation and promoting bone resorption by increased RANKL expression through upregulation of PTHrP expression.[72] The BMD signal in this locus is also observed in GWAS of human stature,[12] providing evidence for pleiotropic effects of the gene and for the hedgehog pathway being involved in both skeletal traits.

3.18 11p11.2 *LRP4* and *ARGHAP1*

The low density lipoprotein receptor-related protein 4 (*LRP4*) gene spans 62 kb and was proposed as a candidate for regulation of total hip BMD by a GWAS metaanalysis based on Icelandic (deCODE genetics) and Northern European populations,[20] which identified

variants mapping close to the gene associated at suggestive level. A subsequent effort in five Northern European populations found variants in the locus associated at GWSL.[23] Nevertheless, the GWAS signals map within a region of high LD containing several genes including the Rho GTPase activating protein 1 (*ARHGAP1*) gene and the coagulation factor II (*F2*) gene. At the moment it cannot be determined, among them which of the gene is responsible for the reported associations. Regarding the candidacy of *ARGHAP1*, it is known that small GPTases, such as Rho play an important role regulating bone cell activity while *LRP4* is homologous to *LRP5*, which is known to regulate BMD (see Section 3.21). Further work will be required to define the functional mechanisms underlying the associations that have been reported in this genomic region.

3.19 11p14.1 *DCDC5*, *LIN7C*, and LGR4

The doublecortin domain containing 5 (*DCDC5*) and 1 (*DCDC1*) are big genes spanning 297 and 427 kb in this locus, which were initially proposed as possible candidates for regulation of lumbar spine BMD as they were the closest genes to the GWAS signal identified by the first GEFOS GWAS metaanalysis.[23] However, neither gene appears to be highly expressed in bone and subsequent efforts have provided more likely candidates to underlie the associations. In the second GEFOS metaanalysis two independent signals were identified in this locus, one close to *DCDC5* and the other mapping to *Lin-7 Homolog C (LIN7C)*. The latter associated variant constituted a strong so-called expression quantitative trait locus (eQTL) of *LIN7C*. The *LIN7C, crumbs cell polarity complex Component* (*LIN7C*) gene spans 12 kb and functions establishing and maintaining the asymmetric distribution of channels and receptors at the plasma membrane of polarized cells. Variants mapping to the gene have in addition been associated with pediatric BMD postulating an effect on skeletal development.[66]

The *LGR4* gene spans 106 kb and encodes a G-protein coupled receptor that binds R-spondins involved in the WNT and mTOR signaling pathways. A variant (c.376C > T) mapping to this locus was identified in a WGS effort in a sample of Icelandic individuals[73] composed of 4,931 individuals with low BMD and 69,034 controls. The T allele was shown to have a large effect on (decreasing) BMD and was associated with increased risk for osteoporotic fractures, among other disease outcomes (e.g., cancer and CVD). The mutation introduces a rare nonsense mutation into exon 4, which terminates LGR4 and completely disrupts the function of the protein. Recently, LGR4 has been shown to act as a second RANKL receptor that negatively regulates osteoclast differentiation and bone resorption.[74]

3.20 11p15.1 *SOX6*

The SRY (sex determining region Y)-box 6 (*SOX6*), which is a large gene spanning 773 kb, emerged as a candidate for regulation for BMD in the first GEFOS GWAS metaanalysis on populations of European descent, showing a genome-wide significant association signal with femoral neck BMD, situated 297 kb upstream from SOX6.[23] Both *SOX5* and *SOX6* encode transcription factors which play essential roles in chondrocyte differentiation and endochondral ossification, are needed for growth plate multilayered establishment and for proper and timely development of endochondral bones.[75] Therefore, genetic variation in this candidate gene may well be mediating skeletal development and influencing BMD variation at the population level.

3.21 11q13.2 *LRP5*

The *LRP5* gene spans 137 kb and has for decades been known to play an important role in bone biology. Its role as a critical regulator of bone mass was first established by linkage studies[76–78] identifying mutations in the gene as producing the *osteoporosis–pseudoglioma* syndrome[76,79,80] and several presentations of the *high bone mass* syndromes[81–84] including autosomal dominant forms of *type 2 van Buchem disease*[83] and *osteosclerosis/endosteal hyperostosis*.[84]

Genetic variants in the *LRP5* locus have been identified as significant determinants of BMD by several of the GWAS to date[18,23,85] but, interestingly, not in those associations arising from Icelandic populations.[19,20,68] Even though several early candidate gene association studies showed that common variants in *LRP5* underlie variation of BMD in the general population, it was only in the GENOMOS consortium including ≈45,000 individuals (the largest candidate gene study run to date), that two nonsynonymous coding variants were robustly associated with BMD, at a level of significance surpassing the current stringent standards of genome-wide level. An association with risk of fracture was also observed in that study and has been recently confirmed in the latest effort of the GEFOS consortium.[26] Many common *LRP5* variants have been studied in association studies, but the most likely functional candidates are a valine to methionine variant in exon 9 at codon 667 (V667M) and an alanine to valine substitution at position 1330 (A1330V) in exon 18 which are also precisely tagged by the GWAS signal.

3.22 12q13.12 *DHH*

The *Desert Hedgehog* (DHH) gene is a relatively small gene spanning 3.4 kb, which encodes a protein member of the hedgehog gene family of signaling molecules that play

an important role in regulating morphogenesis. DHH exerts an intercellular signal essential for a variety of patterning events during development; also in interaction with HHIP, PTCH1 (see 9q22.32 earlier**)** and PTCH2. Variants in this locus were identified as associated at GWSL with lumbar spine BMD in the second GEFOS metaanalysis.[26]

3.23 12q13.13 SP7

SP7 is a small gene spanning 19 kb which encodes Osterix, a transcription factor exerting an essential role in osteoblast differentiation.[86,87] Variation in the gene was first reported to influence BMD variation in an Icelandic population followed by a report showing its effect on peak bone mass accrual in children from the United Kingdom.[21] Since then, several GWAS have shown association with variants mapping in the SP7 region.[23,25,26] Additional studies are required to investigate the mechanisms underlying these associations with BMD. Noteworthy is the fact that a homozygous single basepair deletion in SP7 has been shown to cause *Type IX osteogenesis imperfecta*.

3.24 13q14.11 *TNFSF11* (RANKL)

The tumor necrosis factor (ligand) superfamily member 11 gene spans 45 kb and encodes RANKL, a member of the TNF superfamily which stimulates bone resorption by activating RANK signaling. Variants in *RANKL* have been consistently associated with lumbar spine BMD[20,23,25,26] since the first report arising from Icelandic populations (deCODE genetics).[19] In a recent report based on peripheral quantitative computed tomography, variants in this locus distinct from those associated with DXA BMD (i.e., not in LD), were associated at GWSL with volumetric and cortical bone density in 999 UK adolescents and 935 young Swedish adults.[88] These findings imply that allelic heterogeneity may be governing the associations with areal and volumetric BMD seen in this locus. Even though the involvement of the OPG-RANK-RANKL pathway in bone metabolism is well established,[69] the exact functional mechanisms by which genetic variation in these genes regulate BMD remains to be investigated.

3.25 14q32.32 *MARK3*

This gene has a size of 119 kb and encodes MAP/microtubule affinity-regulating kinase 3, a member of the AMP kinase superfamily of proteins.[89] Variants in this gene were first reported as associated at GWSL with total hip BMD in an Icelandic population after replication in other European populations.[20] In the first GEFOS metaanalysis on femoral neck and lumbar spine, variants in MARK3 were significant but not at GWSL.[23] Nonetheless, variants in this locus did achieve a GWSL in the most recent effort involving an expanded set of populations of European descent and including East-Asian cohorts.[26] Mechanisms, by which variations in this gene might affect BMD and/or bone physiology, are yet to be described.

3.26 16p13.3 CLCN7 and AXIN1

The chloride channel 7 is a gene spanning 31 kb and coding for ClC-7. CIC-7 is particularly important for regulating pH and needed for the adequate resorptive function of osteoclasts through lysosomal acidification. CLCN7 mutations cause (infantile malignant) *type 4 autosomal recessive osteopetrosis* (OPTB4), and (Albers–Schonberg) *type 2 autosomal dominant osteopetrosis* (OPTA2), also called "marble bone disease." In contrast, the *AXIN1* gene which spans 74 kb encodes a cytoplasmic protein containing G-protein signaling regulation, dishevelled and axin domains. The encoded protein is a component of the β-catenin destruction complex required for regulating CTNNB1 levels through phosphorylation and ubiquitination, which is critical for modulating Wnt-signaling. Variants in this locus were first reported as suggestive of association with total hip, femoral neck, and lumbar spine BMD in Australian populations,[25] while variants in this locus reached genome-wide significance in the second GEFOS metaanalysis of GWAS.[26] Recently, in an Icelandic study[32] a new low frequency signal (frequency of 3.5%) mapping to *AXIN1* was found strongly associated with lumbar spine BMD, but much weaker with hip BMD and fracture. This novel signal was independent from (i.e., not tagged by) the previously reported common SNP (frequency of 43%) in this locus.[25,26]

3.27 16q24.1 *FOXL1* and *FOXC2*

This locus was first identified in the first GEFOS metaanalysis in European populations as containing variants associated with lumbar spine BMD.[23] The region contains a cluster of small genes of the "forkhead" (or winged helix) FOX gene family that are part of the TGFβ pathway and are involved in organogenesis. The GWAS signal maps close to the forkhead box L1 (*FOXL1*) gene of size 5.8 kb and the forkhead box C2 (*FOXC2*) gene spanning 2.5 kb. *FOXL1* is involved in the regulation of multiple processes including metabolism and cell proliferation. Changes in FOXL1 expression have been associated with ontogenesis and diverse oncogenic processes (including osteosarcoma).[90] Mutations in *FOXC2* have been reported as causing the lymphedema–distichiasis syndrome,[91,92] a disorder characterized by lymphedema of the limbs coupled with various other features but no evident skeletal phenotype. Nevertheless,

Foxc2-deficient mice exhibit aortic arch anomalies and defects of skeletogenesis in the craniofacial bones and vertebral column,[93] while inactivating mutations affecting the FOX gene cluster involving *FOXC2* can cause severe malformations of the VACTERL (vertebral, anal, cardiac, tracheoesophageal, renal, and limb) type in humans.[94] Further, *FOXC2* expression is regulated by bone morphogenetic proteins[95] and can effect osteoblast differentiation of mesenchymal cells through activation of canonical Wnt-β-catenin signals[96] and via upregulation of integrin beta 1.[97]

3.28 17q12-22 *CRHR1*

This locus was identified by the first GEFOS GWAS metaanalyses on populations of European descent proposing corticotropin-releasing factor receptor 1 (*CRHR1*) as the closest candidate gene to be influencing BMD variation at the population level.[23] *CRHR1* is a gene that spans 214 kb coding for a member of the corticotropin-releasing factor family of G-protein-coupled receptors that binds neuropeptides involved in regulation of the hypothalamic-pituitary-adrenal pathway. Nevertheless, the involvement of *CRHR1* on this BMD signal cannot be unequivocally determined considering that the GWAS signals lie in a region of very high LD containing many genes. The high LD in the region arises from a common inversion polymorphism spanning about 900 kb, which has been subject to positive selection and which has been highly preserved for more than 3 million years in humans of Northern European descent.[98]

3.29 17q21.31 *SOST* and *HDAC5*

The first GWAS report identifying variants mapping to this locus was based on a discovery setting based on Icelandic individuals. In that study, variants mapped close to an open reading frame sequence (*C17orf53*) and to a 5 kb gene encoding sclerostin. A nonsynonymous variant in *C17orf53* was associated with hip BMD but not at GWSL, while the marker mapping in the vicinity of *SOST* did achieve GWS. Sclerostin is a protein produced by osteocytes that inhibits bone formation by blocking the binding of members of the Wnt family to the LRP5 receptor.[99] This way, *SOST* arises as an excellent candidate for genetic regulation of BMD considering that inactivating mutations of *SOST* cause sclerosteosis and mutations in a downstream enhancer cause van Buchem's disease.[100,101] Variants mapping to *SOST* did not achieve genome-wide significance in the first GEFOS metaanalysis with lumbar spine or femoral neck BMD.[23] Nevertheless, a metaanalysis using a candidate gene approach examining 150 genes on the same exact datasets did identify variants mapping to *SOST* as significantly associated with BMD,[102] while the second GEFOS metaanalysis has now identified variants mapping to *SOST* as associated at GWSL with BMD.[26] Very recently, a new and independent signal in the SOST locus has been reported. A variant (frequency 9.6%) was associated with increased hip and lumbar spine BMD and with lower risk for nonvertebral fractures and particularly a very strong association with vertebral fractures.[32] Further, the first GEFOS metaanalysis also identified variants as associated with FN-BMD at GWS level in this locus, but mapping in an intronic region of the histone deacetylase 5 (*HDAC5*) gene[23] on what appears to be an independent signal from that mapping to *SOST* and the same one arising from *C17orf53*. At present, it is not possible to determine from which gene this signal is arising as the function of *C17orf53* is unknown. On the other hand, *HDAC5* is a 47 kb gene which is ubiquitously expressed and involved in transcriptional regulation through MEF2 inhibition[103] and involved in muscle differentiation.[104]

3.30 18q21.33 *TNFRSF11A*

The tumor necrosis factor receptor superfamily member 11a (*TNFRSF11A*) is a 66 kb gene that encodes the receptor activator of the NF-κB or RANK protein. The RANK receptor is expressed in osteoclasts and plays a critical role in regulating osteoclast differentiation and function, constituting a key element for the stimulation of bone resorption. Mutations in the RANK gene (Chapter 26) are responsible for the osteoclast-poor form of autosomal recessive osteopetrosis associated with hypogammaglobulinemia (OPTB7).[105] The first study to identify common variants in the gene associated at GWSL was on Icelandic populations[20] and subsequently consistently replicated.[23,26]

3.31 20p12 *JAG1*

The first report identifying variants mapping close to *JAG1* as associated with lumbar spine BMD came from Asian populations[24] but has been replicated in the second large-scale metaanalysis of the GEFOS consortium, including populations of both European and Asian descent.[26] Jagged-1 is a ligand of the Notch receptor that, after binding, triggers the release of the intracellular unit of the Notch receptor from the membrane. This unit translocates to the nucleus and activates transcription factors key for cell differentiation and morphogenesis processes.[106] In 2008, two reports in mice showed that Notch signaling stimulates early proliferation of osteoblastic lineages and that when knocked down results in an osteoporosis-like phenotype.[107,108] In addition, *JAG1* may be mediating some anabolic responses considering that PTH stimulation of osteoblast results in expression of *JAG1*.[109]

4 GWAS IN OTHER ETHNIC GROUPS AND FOR OTHER OSTEOPOROSIS PHENOTYPES

It is now clear that BMD is the trait producing the largest yield of identified loci probably due to the combination of favorable properties including its high heritability, measurement precision, and widespread use (allowing collections of samples of large size). Several other genes like *IL21R*,[110] PTH,[110] *ALDH7A1*,[111] *TBC1D8*,[67] *OSBPL1A*,[67] *RAP1A*,[67] *PLCL1*,[112] *RTP3*,[113] *ADAMTS18*,[114] and T*GFBR3*[114] have been postulated as involved in diverse osteoporosis traits including BMD,[110,114] bone geometry,[112,113] fracture,[111] and derived phenotype combinations.[67] Nevertheless, several of the latter reported associations are under or just over the genome-wide significance threshold, are based on small and heterogeneous groups of individuals, or have not been replicated to date in well-powered settings summing several tens of thousands of individuals.[26,27] Even though population-specific findings are a possibility, it has recently been shown that the vast majority of GWAS associations replicate across populations of different ethnic backgrounds.[68] Additional replication is needed to consolidate these findings as robust candidate genes of osteoporosis.

The largest GWAS metaanalysis for skeletal phenotypes to date was recently reported by investigators using data from the UKBiobank Study in relation to estimated BMD from heel ultrasound measurements in 142,487 individuals of European decent.[28] This GWAS effort identified 307 independent SNPs from 203 loci jointly explaining 11.8% of the variation in heel ultrasound estimated BMD. Such a large study highlights the value of expanding the sample size of GWAS and the great potential for identifying new biology as a next step toward driving these discoveries in direction of clinical applications (e.g., risk stratification and identification of new drug targets and indications).

5 CONCLUSIONS

To date at least 70 loci have shown robust association with BMD containing an even larger number of candidate genes potentially underlying these GWAS signals. Here, we have reviewed a subset of them, mainly those common variants that have been reported in the literature associated at GWSL and been replicated, together with the more recent discoveries of rare and low-frequency variants whose identification has been facilitated by the emergence of whole-genome sequenced reference sets. Interestingly, many of these variants map in the vicinity of genes of unknown function (representing cutting-edge new biology), while several other factors

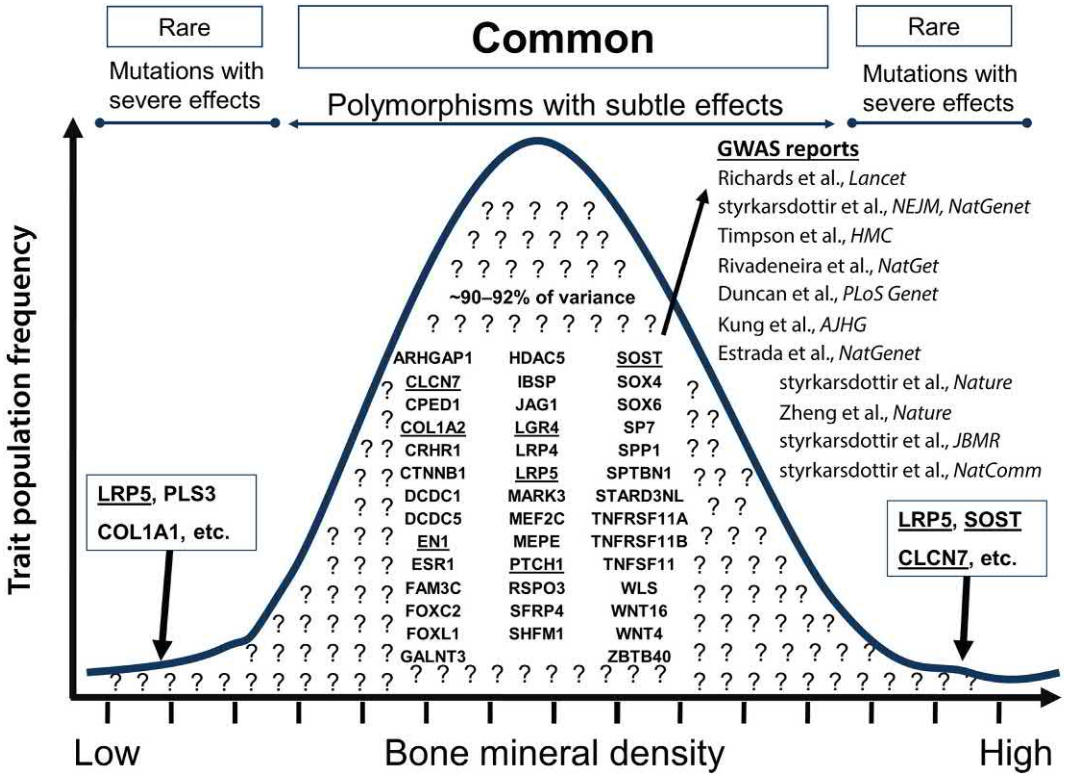

FIGURE 22.3 **Genetic architecture underlying BMD variation.**

cluster within critical biological pathways[115] relevant for bone biology like Wnt signaling, OPG-RANK-RANKL, mesenchymal cell differentiation and the Indian hedgehog pathways. Thus far, these genes identified by GWAS incorporate variants which together explain 8%–10% of the variation in BMD (Fig. 22.3). This suggests that both common and rare variation underlies the genetic architecture of BMD and many more genetic determinants are yet to be identified. While the objective is to keep scrutinizing variants particularly in the low frequency and rare allele spectrum, sequencing studies are not expected to become much bigger considering the still costly investments and computational requirements needed. In contrast, GWAS based on arrays: (1) have become increasingly affordable, (2) contain rare and clinically relevant variants, and (3) will soon reach many millions of samples. Therefore, it is expected that future GWAS will continue to advance the field of complex diseases, osteoporosis included. One of the expectations from genetic studies of common diseases like osteoporosis is the possibility to improve risk prediction (i.e., as compared to what is currently possible with the use of clinical factors and/or biomarkers like BMD). Nevertheless, this goal is not yet feasible to achieve since the small fraction of phenotypic variance explained still provides little added value in terms of prediction; hence, limiting at the moment any such derived clinical utility. From a distinct clinical perspective (i.e., translation to therapies), GWAS have pinpointed many factors in critical molecular pathways (e.g., Wnt signaling), as candidates for therapeutic applications. Such potential is highlighted by the identification (among others) of genes encoding proteins that are currently subject to mediate novel bone medications. This is the case for denosumab, a human monoclonal antibody against RANKL, which is a protein inhibiting bone resorption. Yet even more interesting is the identification of several factors, which can constitute targets for true bone-building drugs, exemplified by the identification of the sclerostin gene for which an anti-sclerostin antibody is in clinical trials. Hence, from the perspective of their therapeutic potential, translation of the GWAS discoveries into clinical applications is an upcoming reality.

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CHAPTER

23

Osteogenesis Imperfecta

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1 INTRODUCTION

Osteogenesis imperfecta (OI) is one of the two most common inherited skeletal dysplasias, causing significant morbidity and mortality to affected individuals. Also known as "Brittle Bone Disease," OI is a group of conditions, which shares an etiology related directly or indirectly to type I collagen. The most common clinical features of OI include bone fragility and deformity, and growth deficiency. Additional variable symptoms associated with OI include blue sclerae, hearing loss, dentinogenesis imperfecta (DI), pulmonary deficiency, and cardiac valvular dysfunction.[1] Phenotypic severity is extremely variable and ranges from lethality in the perinatal period to milder cases that present as an osteoporotic condition with increased susceptibility to fractures. OI detectable in infancy occurs with an estimated incidence of approximately 1 in 15,000–20,000 births.[2–5]

Understanding of the genetic basis of OI has expanded rapidly since 2005, as causative genes for recessive OI were identified; extensive research is ongoing to dissect the cellular and extracellular mechanisms leading to bone dysplasia.[6] The original Sillence Classification, which preceded the identification of the molecular causes of OI, is based on genetic, radiological, and clinical criteria.[2] The four types recognized in this classification (types I–IV) have since been attributed to autosomal dominant mutations in *COL1A1* or *COL1A2*, which directly alter the quantity or primary structure of type I collagen. However, understanding of OI as a collagen-related disorder has allowed for a more specific description of OI into 18 functionally related subtypes based on clinical features, bone histology, inheritance patterns, and genetic causes.[7] Thus, OI is now classified according to histological distinctions and phenotypic features (types V and VI),[8,9] autosomal recessive inheritance patterns of mutations in genes encoding proteins involved in posttranslational modification (types VII–IX)[10–12] or processing and crosslinking (types X–XII)[13–15] of collagen, and defects of osteoblast differentiation and function (types XIII–XVIII).[16–21]

OI, which literally means imperfect bone formation, is a disorder of endochondral bone ossification. With at least 17 distinct gene defects now attributed to OI, a shift from gene identification to investigation of the alterations in common intra- and extracellular pathways affecting bone quality is underway. Bone is formed and remodeled by coordinated efforts of three different cell types: osteoblasts, osteoclasts, and osteocytes.[22] Bone-forming osteoblasts are mesenchymally-derived cells whose primary functions are the secretion and mineralization of the bone extracellular matrix (ECM), which includes both type I collagen and numerous collagen-interacting matrix proteins believed to regulate mineralization including proteoglycans, glycoproteins, γ-carboxyglutamic acid-containing molecules, and phosphoproteins. Mature osteoclasts are multinucleated cells of hematopoietic origin, derived from bone marrow mononuclear precursors of the monocyte-macrophage lineage, whose primary role in bone is to resorb existing bone tissue. Cellular crosstalk between osteoblast and osteoclast lineage cells, particularly through the RANK/RANKL/OPG axis, is considered a major regulatory pathway that controls the processes of bone resorption and replacement, referred to as bone remodeling. Osteocytes, which comprise over 90% of the cell population in adult bone, are terminally differentiated osteoblasts, which have become surrounded by and embedded within the mineralized bone matrix. There is increasing evidence to suggest that these cells are intricately involved in orchestrating bone remodeling.[23] Studies on the pathogenesis of diseases, such as OI lead to valuable insights into the dynamics of normal bone development and remodeling.

Although noncollagenous proteins (NCPs) comprise approximately 10%–15% of total bone protein content,

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type I collagen is the primary organic constituent of the ECM that also includes trace amounts of type III, V, and fibril-associated collagens.[24] The type I procollagen monomer is a heterotrimer consisting of two pro-α1(I) and one pro-α2(I) chains encoded by the *COL1A1* and *COL1A2* genes, respectively.[25] Each gene contains a large number (51 and 52, respectively) of small exons, which likely arose by duplication, and which contain a discrete number of codons (usually 18, sometimes 36) beginning with a glycine. Together they encode the core helical domain containing 1014 residues, comprising 338 uninterrupted Gly-Xaa-Yaa triplet repeats, as well as the N- and C-terminal globular prodomains that flank the helix. Within the helical region there is a requirement for glycine at every third residue, since only its small side chain can be accommodated in the sterically restricted interior aspect of the triple helix.[26] Stabilization of the triple helical structure is provided by the X- and Y-position residues, which are often proline and 4R-hydroxyproline, or by the formation of interchain salt bridges that form between positively charged Y-position lysyl residues and negatively charged X-position glutamic/aspartic acid residues.[27,28] The combination of hydrogen bonds, electrostatic interactions, and hydrophobic forces create a rigid, yet flexible, rod-like structure perfectly suited to provide support and organization to the ECM.

Individual preprocollagen α-chains are synthesized into the rough endoplasmic reticulum (ER), where about half of proline and a quarter of lysine residues in the helical portion of procollagen are modified by hydroxylation, followed by glycosylation of some hydroxylysyl residues. Helical hydroxylation is catalyzed by three different enzymes: prolyl 3-hydroxylase 1, prolyl 4-hydroxylase, and lysyl-hydroxylases.[29] The substrate sequence for each enzyme is very specific, and comprises Pro–4Hyp–Gly, Xaa–Pro–Gly, and Xaa–Lys–Gly, respectively (hydroxylated residues underlined). Prolyl 4- and lysyl-hydroxylation occur throughout the length of the helix, but prolyl 3-hydroxylation occurs only at specific residues, namely Pro986 in α1(I) (completely hydroxylated) and Pro707 in α2(I) (80% hydroxylated).[30] Glucosyl–galactosyl transferases are subsequently involved in the glycosylation of some hydroxylysines to form galactosyl–hydroxylysine and glucosyl–galactosyl–hydroxylysine residues. C-propeptide N-linked glycosylation also occurs at specific asparagine residues in the sequence Asn-Ile-Thr of both the proα1(I) and proα2(I) chains.[31]

Correct folding and helical assembly are dependent on the accuracy of these cotranslational modifications, and are facilitated by numerous ER resident chaperones, such as BiP and HSP47. These chaperones have a role in preventing premature folding of the protein, as well as preventing protein aggregation and detecting unfolded or misfolded proteins. However, heterotrimer formation is initiated by a specific chain selection and alignment region in the globular C-propeptide domain, and further stabilized by the formation of disulfide bridges between specific cysteine residues.[32–34] Trimerization and helix folding occur concomitantly with ongoing chain modification, and proceeds along the helical domain toward the N-terminus. Upon completion of helical folding, the residues within the helical region are no longer available for modification.[29]

Modified, folded procollagen monomers are shuttled from the ER to the Golgi in "megacarriers", mediated by the interaction of collagen-specific cargo receptors (TANGO-like proteins), COPII component Sec23, SEDL, and Sar1-GTP, then transported to the cell surface and released into the pericellular space via "collagen condensation vacuoles".[35,36] Once the procollagen monomer is exported into the ECM, mature collagen is formed by the cleavage of the N- and C-terminal propeptide domains by metalloproteinases ADAMTS-2 and BMP1/mTLD-like protein family members, respectively.[37] Finally, the secreted and processed monomers self-assemble into higher order structures, known as heterotypic fibrils, through self-association and interaction with noncollagenous ECM components. The fibrils are stabilized by inter- and intrachain covalent crosslinks between specific domains. As viewed by electron microscopy, heterotypic type I collagen fibrils appear long and cylindrical with a cross-striated periodic banding pattern due to lateral assembly of monomers with a longitudinal displacement of approximately 234 amino acids, commonly referred to as the "quarter-staggered array".[38]

2 CLINICAL DESCRIPTION

The clinical spectrum of OI is extremely broad, ranging from perinatal lethal bone dysplasia to barely detectable conditions. To have a general correlation of clinical features and genetics, a classification system is required. Since 1979, the Sillence Classification, based on clinical and radiographic criteria, has proven useful to clinicians and researchers alike. With the rapid discovery of additional genes responsible for the rare recessive forms of OI, several alternative classifications have been proposed.[6,39,40] We favor a classification based on the genetics of OI, which assigns the Sillence numeration to cases with primary defects in type I collagen, and then extends this numeration for types with defects in molecules that interact with type I collagen (Table 23.1).

From a clinical standpoint, this groups together children with a common etiology of their condition and conveys general severity, gene defect, and inheritance pattern with a single term. Despite some variability in severity in the same individual over time or between related individuals, the designation of OI type would not change with age or between family members. This is an important clin-

TABLE 23.1 Clinical Description of Osteogenesis Imperfecta (OI)

OI type	Gene (protein)	Inheritance	Severity	Clinical description
Defects in collagen synthesis and structure				
I	*COL1A1* (Type I collagen)	AD	Mild	Always associated with blue sclerae which persist throughout life. Patients attain normal or near normal stature with a relatively mild increase in susceptibility to long bone fracture. Vertebral compression fractures commonly occur, as does premature hearing loss with advancing age. Accounts for about 50% of all OI cases.
II	*COL1A1/ COL1A2* (Type I collagen)	AD	Lethal	Lethal in utero or perinatally. Sixty percent of affected infants surviving birth die within the first 24 h, and 80% die within the first week. Mortality, likely results from multiple rib fractures leading to respiratory insufficiency, as well as inherent malformation of the thorax, lungs, and other connective tissues. Long bones are short, severely bowed, and break easily in utero, and the entire skeleton is poorly mineralized and underdeveloped. Sclerae are blue or gray. Usually arise as a result of de novo mutations, but parental mosaicism generally accounts for recurring cases.
III	*COL1A1/ COL1A2* (Type I collagen)	AD	Progresses with age	Most severe nonlethal form. Detectable prenatally and at birth. Progressive deforming. Multiple (as many as 200) fractures occur in utero and throughout life. Even in the absence of fractures, overall skeletal deformity progresses with age. Faces often have a characteristic triangular appearance, associated with frontal bossing, blue/gray sclera and DI. Additional skeletal complications include vertebral/basilar compressions and scoliosis. Stature severely affected, with affected individuals often being less than 1m tall. Most individuals are nonambulators.
IV	*COL1A1/ COL1A2* (Type I collagen)	AD	Moderate	Variable phenotype, with more severe cases overlapping from type III OI. Mild DI is often present, as is adult hearing loss. Stature varies, even between affected individuals within families. Generally able to attain household and/or community ambulation.
Defects in bone mineralization				
V	*IFITM5* (BRIL)	AD	Moderate/ severe	Accounts for about 5% of OI cases. All affected individuals have identical recurrent mutation. Moderately severe with highly variable phenotype. Classical triad includes hypertrophic callus, dense metaphyseal band and ossification of interosseus membrane of forearm on radiographs. Also high frequency of dislocation/subluxation of the radial head. Bone lamellation pattern has a distinctive histological appearance described as mesh-like.
VI	*SERPINF1* (PEDF)	AR	Severe	Affected individuals appear normal at birth and have first fracture around 1 year of age. Subsequently, their OI is progressive deforming with many fractures, severe bowing, and scoliosis. Serum PEDF is low; serum alkaline phosphatase elevated in childhood. Bone lamella have a distinctive fish-scale appearance under polarized light. Histology shows broad seams of unmineralized osteoid and prolonged MLT. Sclerae, teeth, and hearing are normal. Affected individual responds poorly to bisphosphonate therapy.
Defects in collagen modification				
VII	*CRTAP* (CRTAP)	AR	Severe/ lethal	Similar in skeletal severity and clinical presentation to types II and III OI. Severe growth deficiency with *coxa vara*, rhizomelia, white sclera, neonatal fractures, and broad long bones, which are undertubulated. Most affected children die during first 5 years of life due to respiratory causes.
VIII	*LEPRE1* (P3H1)	AR	Severe/ lethal	Similar to type VII OI. About half of cases are caused by homozygosity for a West African founder allele, which is almost uniformly lethal. Several individuals with other mutant alleles have been known to attain their 3rd decade.
IX	*PPIB* (CyPB)	AR	Moderate/ lethal	Very few cases reported, of which most are lethal with a presentation similar to types VII/VIII except for absence of rhizomelia. Two cases have been reported with moderate phenotypes, similar to more severe type IV OI. Their sclerae are white, teeth normal, no rhizomelia.

(*Continued*)

TABLE 23.1 Clinical Description of Osteogenesis Imperfecta (OI) (*cont.*)

OI type	Gene (protein)	Inheritance	Severity	Clinical description
Defects in collagen processing and crosslinking				
X	*SERPINH1* (HSP47)	AR	Severe/ lethal	Severe, deforming type similar to type III OI. Three reported patients had variable findings of macrocephaly, high-bossed forehead, midface hypoplasia with shallow orbits, blue sclerae, DI, generalized joint laxity, and scoliosis. Two probands succumbed to pulmonary complications in neonatal period or infancy.
XI	*FKBP10* (FKBP65)	AR	Progresses with age	Moderate to severe deforming OI with joint laxity and scoliosis. Teeth are normal. Affected individuals may or may not have congenital large joint contracture, even in same family. Allelic or contiguous with Bruck syndrome type I in some families. Small deletion in *FKBP10* causes Kuskokwim syndrome, with congenital contractures in large joints and osteopenia with few fractures.
XII	*BMP1* (BMP1/ mTLD)	AR	Severe	Severe bone deformities, osteoporosis, multiple fractures, kyphoscoliosis, and umbilical hernia. Short stature with rhizomelia. Faint blue sclerae, normal teeth. Phenotype overlaps with dominant high bone mass form of OI. Some affected individuals have elevated bone density DXA *z*-score.
Defects in osteoblast differentiation and function				
XIII	*SP7* (OSX)	AR	Severe	Reported in a single child with recurrent fractures, bone deformities, generalized osteoporosis, delayed eruption of teeth without DI, white sclerae.
XIV	*TMEM38B* (TRIC-B)	AR	Moderate/ severe	First identified as founder mutation in Bedouin populations. Moderately severe bone phenotype with bowing and fragility of long bones. Sclerae may be blue. Normal teeth and hearing.
XV	*WNT1* (WNT1)	AD/AR	Mild to severe	Heterozygosity results in osteoporosis; homozygosity causes severe OI with progressive bone fragility, long bone deformity, osteopenia, short stature, and kyphoscoliosis.
XVI	*CREB3L1* (OASIS)	AR	Severe	Reported in two siblings. One had multiple fractures of extremities. Second sibling showed thin ribs and fracturing of bowed humeri and femora at 19-weeks gestation.
XVII	*SPARC* (osteonectin)	AR	Moderate/ severe	Two affected individuals reported, who were normal at birth and then developed severe bone fragility and kyphoscoliosis with vertebral compressions, muscle hypotonia, and joint hyperlaxity.
XVIII	*MBTPS2* (S2P)	XR	Moderate/ severe	X-linked inheritance; absence of symptoms of IFAP/KFSD; prenatal fractures of ribs and long bones, bowing of upper and lower extremities, generalized osteopenia, kyphoscoliosis, pectal deformities, and short stature.
Unclassified osteogenesis imperfecta-like or collagen-based disorders				
	PLOD2 (LH2)	AR	Moderate/ severe	Bruck syndrome type 2 with joint contractures.
	LRP5 (LRP5)	AD	Mild/ moderate	Osteoporosis-pseudoglioma syndrome, osteosclerosis, osteopetrosis type I.

DI, Dentinogenesis imperfecta.

ical feature, since intrafamilial variability is quite common in OI. From a research standpoint, children with the same bone dysplasia mechanism are classified together, encouraging unified insights. Alternative classifications are based on clinical severity and group all of the rare types of OI as subtypes within the original Sillence Classification. Ultimately, this predominantly clinical classification is confusing for gene etiology and phenotype progression, and sometimes changes the type designation of the same individual as they age, or of members of the same family with phenotypic variability.

OI types I–IV are caused by defects in the two genes encoding the major bone protein, type I collagen, *COL1A1*, and *COL1A2*. The great majority of OI cases fall into these types. Type I OI (OMIM #166200) is the mildest form of OI, in some cases appearing like early-onset osteoporosis. Most individuals with type I OI have onset of fractures after they attain ambulation. Their fractures heal without

bone deformity. These individuals often have blue sclerae, onset of hearing loss in the 3rd decade, easy bruising and joint hyperextensibility. The absence or presence of DI divides type I OI into subtypes IA and IB.[41]

Type II OI (OMIM #166210) cases are the most severe, and are lethal in the perinatal period, stillborn, or hydropic. The skeleton is extremely fragile in type II OI and almost all cases have multiple in utero fractures of both extremities and rib cage, which are detectable prenatally. Birth weight and length are decreased; the undermineralized skull is soft with large anterior and posterior fontanels. The thorax is narrow; and the short lower extremities are held in the frog leg position. The scleral hue is usually a deep blue-gray. Those infants who survive the perinatal period often die in the first year of life from pneumonia or respiratory insufficiency.

Type III OI (OMIM #259420), the progressive deforming variety, overlaps in clinical presentation at birth with the milder cases of type II OI in terms of in utero fractures and deformity, undermineralized skeleton, and large soft cranium. As these children grow, the long bones are markedly osteoporotic and undertubulated (lacking diaphyseal modeling), with bowing deformities even in the absence of fractures. Vertebrae are often compressed, leading to scoliosis. They have extreme short stature, with final height in the range of a prepubertal child; half of these children have radiographic "popcorn" at the epiphyses. While type III OI is compatible with survival beyond the 4th decade, a substantial percentage of individuals succumb to pneumonia or other respiratory illnesses and many adult survivors develop *cor pulmonale*.

Type IV OI (OMIM #166220), the moderately severe form, may be detectable at birth with perinatal fractures or may present after ambulation. These children tend to have several fractures per year and moderate bowing of long bones. They have platyspondyly and may develop scoliosis. Most type IV OI individuals attain ambulation, often with the assistance of intramedullary rods in long bones. They may have white or blue sclerae, and may or may not have DI. Final adult stature ranges from the average height of a midteenager to near normal; about a quarter of cases have popcorn epiphyses.

Type V OI (OMIM #610967) has moderate skeletal severity but is distinguished by a triad of findings: a dense metaphyseal band and ossification of the interosseous membrane, both detected on radiographs, and the development of hypertrophic calluses at fracture sites. These individuals have white sclerae, normal dentition, and "mesh-like" lamella on bone histology. Subsequent to the identification of the genetic cause of type V OI as a recurrent mutation at the 5′-end of *IFITM5*/BRIL (c.-14C>T), the variability of expression of the clinical triad has been appreciated as many patients were identified who did not have hypertrophic callus, ossification of the interosseous membrane or radial head dislocation.[42,43]

Types VI–XVIII OI are rare recessive forms which fall into four functional groups: those with mineralization defects (type VI OI, which shares a pathway with dominantly inherited type V OI), those with collagen modification defects (types VII–IX), collagen chaperone defects (types X–XI) or processing defects (type XII), and forms with defects in osteoblast differentiation and function (types XIII–XVIII). Type VI OI (OMIM #613982) is difficult to distinguish without bone histology, which is characterized by absence of the birefringent pattern of normal lamellar bone under polarized light, often with a fish-scale appearance, and high levels of unmineralized osteoid without increased remodeling.[9] Skeletal severity is undetectable to moderate in infancy, but progresses to severe OI, with disorganized bone tissue and scoliosis. Teeth are normal and sclerae are normal or faintly blue. Null mutations in *SERPINF1* have been identified in patients with this form.[44]

Types VII, VIII, and IX are each caused by deficiency of a component of the collagen prolyl 3-hydroxylation complex.[10–12,45–48] Because types VII and VIII OI (OMIM #610682; #610915) are caused by the absence of mutually protective proteins (CRTAP and P3H1, respectively), absence of either protein leads to absence of both, and their phenotypes are substantially overlapping. These infants have severe or lethal OI, with rhizomelia, a disproportionate shortening of the proximal segment of the limbs. They have white sclerae, and a normal or small head circumference. Some type VII infants have had extraskeletal manifestations in the heart or kidneys.[45,46] Although type VIII OI is most commonly caused by an almost uniformly lethal founder allele from West Africa, other *LEPRE1* mutant alleles are compatible with survival into the 3rd decade. Surviving children have extreme short stature, scoliosis, bulbous metaphyses, severely undermineralized bone (DXA *z*-score −6 or −7), rhizomelia, and long, gracile hands.[11,46,49] Type IX OI (OMIM #259440) has broader severity, ranging from lethal to moderate skeletal dysplasia. Affected individuals have white sclerae, normal teeth, and hearing, without rhizomelia.[12,47,48]

Types X and XI are caused by deficiency of collagen chaperones, HSP47, and FKBP10, respectively (OMIM #613848; #610968).[13,14,50–53] Two of three probands with HSP47 defects have been characterized with severe skeletal dysplasia, blue sclerae, and DI, as well as atypical extraskeletal features and died in early childhood.[13,54,55] Type XI OI contains Bruck syndrome (OI with congenital contractures) as a subtype. Probands have deforming OI with fractures, vertebral compressions and short stature. Ligamentous laxity and scoliosis have been reported, while sclerae and teeth are normal. Contractures appear to be a variable feature and may or may not occur even with affected siblings.

Type XII OI is caused by a missense mutation in the catalytic protease domain of *BMP1*. The gene defect was

shown to interfere with the extracellular processing of the C-propeptide of type I collagen. The mutation results in skeletal phenotypes ranging from mild to severe and progressively deforming, and may include severe scoliosis, multiple fractures, normal teeth, faint blue sclerae, and umbilical hernias.[15,56–60] Compromised processing of types II and III procollagen, prolysyl oxidase (LOX), as well as other secreted proteins may also contribute to the greater severity of this phenotype compared to mutations in the type I collagen C-propeptide cleavage site.

The most recently delineated grouping of OI types has defects that alter osteoblast differentiation and function. This includes *SP7*, which encodes the protein osterix (OSX), and in which a homozygous frameshift mutation was identified in a proband with moderate OI characterized by recurrent fractures, decreased bone density and mild deformities, delayed tooth eruption, and normal hearing and sclerae.[16] Unfortunately, no cells were available to characterize the biochemical consequences of this mutation.

Deficiency of TRIC-B, encoded by *TMEM38B*, is unique from other forms of OI in that pathophysiology of bone dysplasia results from dysregulation of intracellular calcium flux.[61] The skeletal phenotype ranges from mild to moderate, even between siblings. Scleral hue, hearing loss, the presence of wormian bones, pseudoarthrosis, and fracture frequency are all variable.[17,61–64] Normal serum levels of alkaline phosphatase, calcium, and phosphate are consistently reported in probands with type XIV OI (OMIM #615066).

Type XV OI (OMIM #615220) is perhaps one of the most variable forms of OI given the range of phenotypes and inheritance patterns. Dominant *WNT1* mutations can result in early onset osteoporosis, while recessively inherited forms cause more severe OI.[18,65] Some patients with *WNT1* mutations demonstrate neurological abnormalities, ranging from mild cerebellar hypoplasia to complete agenesis of the cerebellum.[66]

Patients with defects in regulated intramembrane proteolysis (RIP) typically have severe OI. There are now a total of eight probands from two distinct pedigrees that have been identified with X-linked type XVIII OI, caused by deficiency of site-2 protease (S2P/*MBTPS2*).[21] All affected males experienced prenatal fractures of ribs and/or long bones and presented with bowing of upper and lower extremities and generalized osteopenia. They have kyphoscoliosis, pectal deformities, and short stature. Scleral hue was variable, while DI and hearing impairment were not detected. Deficiency of *CREB3L1*/OASIS (Type XVI OI, OMIM #616229), a substrate for S2P, has been identified in two siblings from a consanguineous Turkish family.[19] The affected child that survived to term showed beaded ribs, callus formation, multiple fractures of long bones, recurrent pulmonary infections, and cardiac insufficiency before succumbing at 9-months of age.

Two unrelated OI probands were reported with homozygous missense mutations in *SPARC*, which encodes the collagen-binding matrix protein osteonectin.[20] Osteonectin also binds hydroxyapatite and promotes collagen mineralization. Both type XVII OI (OMIM #616507) probands were apparently normal at birth and did not experience their first fractures until 15 and 60 months, respectively. Serum biochemistry was normal for both patients. Consistent findings between both probands include joint hyperlaxity, kyphoscoliosis, and vertebral compression fractures.

3 GENETIC DESCRIPTION

The great majority of OI cases (~80%–85%) have a heterozygous mutation in type I collagen and autosomal dominant inheritance. Often, an affected child is the first OI case in the family. These de novo mutations are quite frequent and likely related to the high-CpG content of the collagen coding sequence.[67] In these cases, the recurrence risk to the parents is no greater than the incidence of OI in the general population. In a substantial proportion of cases, especially at the milder end of the OI skeletal spectrum, an affected child will have one parent with OI. It is not uncommon to see extensive pedigrees, testifying to satisfactory management of the medical aspects of OI and the social integration of affected individuals.

In about 10% of OI cases with a mutation in type I collagen and apparently unaffected parents, the child's OI will have been the result of parental mosaicism.[68] In these cases, the collagen mutation began in the mosaic parent after conception, so that they have populations of normal cells and cells heterozygous for the collagen mutation. If the mutation occurred in cells that contributed to the formation of germ cells, the mosaic carrier may come to medical attention through their child's condition. Most mosaic carriers have never fractured or may have had several fractures in childhood; they may have blue sclerae, mildly short stature, or DI. Some mosaic parents have no soft signs and are confirmed only by detection of their child's mutation in parental leukocyte DNA. To date, parental mosaicism is detectable in leukocyte DNA, although it may be undetectable in a skin biopsy. Interestingly, the proportion of bone cells carrying the collagen mutation in the mosaic parent can be in the 60%–80% range, with minimal symptoms.[69]

Patients with dominant OI have another genetic feature found in many dominant connective tissue disorders, which is phenotypic variability. Phenotype varies significantly, not only between unrelated patients with the same collagen mutation, but also between affected members of the same families. Intrafamilial variation suggests the existence of modifying factors, which modulate the final phenotype. Genetic or environmental

factors affecting bone shape or turnover by osteoclasts, for example, could be involved.

The rare recessive forms of OI, plus dominant type V OI together comprise about 15%–20% of OI cases.[70] To date, many of the genes that cause recessive OI (*CRTAP*, *LEPRE1*, *PPIB*, *SERPINH1*, *FKBP10*, *BMP1*) are involved in the synthesis, posttranslational modification, secretion, or processing of type I collagen. The majority of the recessive cases are at the lethal/severe end of the clinical spectrum and occur in pedigrees with consanguineous marriages.[7] However, relatively common founder alleles occurring in West Africa and among African Americans, in Irish Travelers and among socially isolated First Nations and Alaskan Eskimo tribes cause a substantial proportion of recessive cases.[46,53,71] Types V and VI are defects in mineralization and decrease the quantity of secreted collagen by an unknown mechanism. OI types that are related to collagen through defective osteoblast differentiation (*TMEM38B*, *WNT1*, *SPARC*, *OASIS*, and *MBTPS2*) generally have moderate to severe phenotypes. In families with unaffected parents and two or more affected children it is crucial to distinguish, at the molecular level, between parental mosaicism and a recessively inherited form of OI.

4 MOLECULAR GENETICS

4.1 Defects in Collagen Synthesis and Structure (Types I–IV)

The phenotypic consequences of type I collagen mutations depend on various parameters—by which collagen gene is affected (*COL1A1* or *COL1A2*), the position, and character of the mutation, the effect on mRNA transcription, the consequences for protein formation, modification, and processing, and the effect of the mutant protein in the ECM. The relatively mild phenotype of type I OI is a result of the less severe consequence of *COL1A1* haploinsufficiency. Mutations resulting in a premature termination codon (PTC), with subsequent nonsense-mediated mRNA degradation (NMD) of *COL1A1* mRNA, are estimated to account for around half of all OI mutations. Typical *COL1A1* mutations causing type I OI are point mutations that lead directly to a PTC, or small deletions or insertions that shift the reading frame and lead to a downstream PTC.[72] Interestingly, splice site mutations also cause type I OI. A surprising proportion of splice site mutations lead to use of alternative donors or acceptors within exons or introns, rather than skipping of the full exon. Although the type I collagen molecules that are formed have a normal primary structure, this type of mutation results in synthesis of half the normal quantity of proα1(I) chains, and a milder phenotype.[73,74] Alternatively, shorter transcripts resulting from in-frame deletions cause the formation of dominant negatively acting heterotrimers, and a much more severe phenotype.[75]

The consequences of *COL1A2* quantitative mutations depend on their location. In the *oim/oim* mouse and in one homozygous child, mutations in the *COL1A2* alignment regions result in α1(I) homotrimers, despite the presence of proα2(I) chains in the cells, and cause severe OI.[76,77] *COL1A2* null mutations, in which both transcripts and protein are absent, do not cause OI despite the presence of α1(I) homotrimers. *COL1A2* null mutations cause mild Ehlers–Danlos syndrome (EDS), characterized by childhood joint hypermobility and adult cardiac valve disease.[78,79]

Types II, III, and IV OI are caused by mutations, which cause a structural or functional defect in the collagen type I monomer and have a dominant negative mechanism. A list of published *COL1A1* or *COL1A2* mutations is available from the Osteogenesis Imperfecta Variant Database (http://www.le.ac.uk/genetics/collagen) and, as of August 2016, these mutations numbered 1718 and 969, respectively. The database comprehensively lists the number of mutations found in individual exons and introns of each gene, and the number of each type of mutation. The vast majority of collagen structural mutations are point mutations, which cause glycine substitutions. Of the 338 helical domain glycine residues, about 45% of α1(I) and 43% of α2(I) glycine residues have had at least one substitution described. Many of the glycine substitutions are unique to individual families, although around 25% of substitutions have been described in two or more independent pedigrees, and around 5% have had more than five separate occurrences identified. Substitution of a glycine by any other amino acid causes a delay in helical folding, increasing the time that collagen alpha chains are exposed to ER resident modifying enzymes, resulting in overmodification. The glycine substitutions are distributed fairly evenly along the length of the helix, and can have both lethal and nonlethal outcomes. The phenotype resulting from a particular substitution can be variable, and may depend on the substituting amino acid and the position along the length of the helix, as well as the function of the helical region in which it is located. When different amino acids substitute at the same glycine residue, the severity generally increases when the new residue is charged or, for α1(I), when it contains a branched side chain.

Splice site mutations comprise the second most prevalent variety of structural mutation affecting the collagen helix, accounting for approximately 13% of total point mutations causing OI. Splice site mutations may result in exon skipping, whole intron retention, or utilization of an exonic or intronic cryptic spice site. Due to the exonic arrangement, exon skipping in the helical domain region does not result in a change in the reading

frame. Rather, the alpha chains are incorporated into the heterotrimer, albeit with a register shift, resulting in a dominant negative effect.

Mutations at either end of the collagen helical region, which interfere with the processing of procollagen to collagen, have distinctive phenotypes. At the amino end of collagen, deletion of exon 6, which contains the N-proteinase cleavage site, results in EDS types VIIA and VIIB (OMIM #130060).[80–82] Glycine substitutions or exon skipping within the first 85 residues of the alpha chains are associated with an OI/EDS mixed phenotype.[83,84] This region is highly stable and acts as an anchor for the N-terminal end of the triple helix. The changes are thought to destabilize the amino end of the triple helix and interfere with the processing of the N-propeptide by disrupting the secondary structure of the cleavage site.[85] Incorporation of uncleaved pN-collagen into fibrils results in decreased fibril diameter and compromised matrix integrity. The combination of phenotypes results from two discrete mechanisms: type III or IV OI from the collagen structural mutation, and the EDS symptoms from the inhibition of N-propeptide processing.[83]

Mutations in the C-propeptide cleavage site, which consists of an alanine–aspartic acid doublet in both alpha chains [p.1218–1219 in proα1(I), p.1119–1120 in proα2(I)], result in a high-bone mass phenotype.[86] Biochemical studies for the p.D1219N substitution in proα1(I) and p.A1119T in proα2(I) showed that modification of the collagen is relatively unaffected, but that procollagen processing was severely delayed, resulting in increased amounts of uncleaved procollagen and the presence of pC-collagen (procollagen with the C-propeptide still attached). Incorporation of the pC-collagen into fibrils had an adverse effect on collagen fibril integrity. Clinically, these patients had increased bone fragility concomitant with a paradoxical increase in mineralization density.

Several nonglycine mutations have been described at X and Y positions, which cause other connective tissue phenotypes, such as OI/EDS and Caffey disease (OMIM #114000), plus mild to moderate bone dysplasia.[87–89] The mutations generally involve the substitution of an arginine residue by a cysteine residue, and the formation of intermolecular disulfide bonds within the helical region. In at least one such mutation [p.Arg1066Cys in proα1(I)], disulfide bond formation led to kinking of the collagen heterotrimer, with a consequent register shift, which propagated toward the N-propeptide cleavage site and impaired N-propeptide processing.[87] This substitution resulted in a primarily OI phenotype, with features of EDS. The collagen was slightly overmodified, and fibrils were less dense and had increased diameter compared with controls. Mutations, such as an inframe deletion of a single Gly–Xaa–Yaa triplet also shift the register of the chains in the heterotrimer, but almost invariably lead to a severe or lethal OI phenotype.[90,91]

Another subset of glycine substitutions exerts their detrimental effect by interfering with the binding to collagen of other proteins within the ECM, such as keratin or heparan. Mutations located in the major ligand binding regions MLBR2 and MLBR3 of α1(I) often have a lethal outcome, and in α2(I), lethal mutations are clustered in eight discrete regions along the length of the chain.[75]

In addition to nonsense-mediated degradation of transcripts containing premature stop codons, there are other cellular mechanisms, which may be activated in response to the presence of misfolded or unfolded monomers or trimers. The unfolded protein response (UPR) is initiated when aberrantly folded monomer chains are detected in the ER, and results in ER-associated degradation (ERAD).[92] During ER associated degradation, the chains are retrotranslocated into the cytoplasm, ubiquinated, and degraded by proteasomes. If the mutation still allows trimerization, retrotranslocation is prevented and, instead, an autophagic mechanism is activated.[93] The misfolded trimers form into supramolecular aggregates within the ER, portions of which bud off and become incorporated into an autophagosome where the aberrant proteins are eliminated.

4.2 Bone Mineralization Defects (Types V and VI OI)

Type V OI is the only type in addition to OI types I–IV with an autosomal dominant inheritance pattern.[8] However, type V OI is caused by a single point mutation in the 5′-UTR of the *IFITM5* gene (c.−14C > T), resulting in the addition of five residues (MALEP) to the N-terminus of bone-restricted interferon-induced transmembrane-like protein (BRIL), a transmembrane protein expressed in osteoblasts.[94,95] Type V OI skeletal findings include hyperplastic callus formation, ossification of the forearm interosseous membrane, and dense metaphyseal bands. Studies utilizing patient primary cell lines have suggested that type V OI mineralization has a gain-of-function mechanism, which likely underlies the increased tissue mineralization seen in patients, and is accompanied with decreased type I collagen expression, secretion, and ECM incorporation in osteoblast cultures.[96]

SERPINF1 was identified as the causative gene for type VI OI using whole exome sequencing.[44,97,98] *SERPINF1* encodes pigment epithelium-derived factor (PEDF), a well known antiangiogenesis factor.[99] All *SERPINF1* mutations result in NMD of mRNA transcripts leading to a loss of PEDF expression, but as yet no mechanistic link between lack of PEDF and the prolonged mineralization lag time and decreased mineral apposition rate has been established. PEDF is expressed in a wide variety of tissues, and is secreted into the bone ECM where it binds collagen with high affinity.[100,101] Interestingly, the binding of PEDF to collagen is an essential prerequisite to its

antiangiogenic activity. Patient collagen from cultured fibroblasts has normal modification, processing, and secretion. Based on spatial expression patterns at sites of active bone remodeling in mice, it has been suggested that PEDF is involved in bone homeostasis, and may act in a negative feedback loop to delay osteoclast maturation, such that its absence results in an increase in bone resorption. Patients display high levels of unmineralized osteoid, and a fish scale pattern to bone lamella under polarized light.[9]

Interestingly, an additional *IFITM5* mutation found in a patient with atypical type VI OI has provided evidence that BRIL and PEDF are connected in a novel intracellular signaling pathway that regulates bone mineralization. Three probands clinically diagnosed with severe type VI OI, but without mutations in *SERPINF1*, were found to be heterozygous for the same mutation in *IFITM5*, resulting in p.S40L substitution in the intracellular domain of the BRIL protein.[102,103] *SERPINF1* expression and PEDF secretion is severely reduced in osteoblasts heterozygous for the S40L BRIL substitution, accounting for the type VI OI-like bone histology and serum markers of the patient. Furthermore, the expression profile of osteoblast differentiation markers overlapped reported values in osteoblasts with *SERPINF1* mutations. Complementary data from osteoblasts with a typical type V OI mutation demonstrated increased *SERPINF1* expression and PEDF secretion. Identification of the components of the signaling pathway connecting PEDF and BRIL will shed further light on their roles in bone mineralization.

4.3 Defects in Collagen Modification: The Prolyl 3-Hydroxylase Complex (Types VII, VIII, and IX OI)

Normal posttranslational modification of types I, II, and V collagen includes the 3-hydroxylation of a limited number of proline residues, especially the Pro986 position of both, α1(I) and α1(II) chains. This modification is performed by an ER-resident complex with three components: cartilage-associated protein (CRTAP), prolyl 3-hydroxylase 1 (P3H1), and cyclophilin B (CyPB) which assemble in a 1:1:1 ratio.[10] The complex also has a collagen chaperone function.[104] In addition, independent functions have been described for the forms of CRTAP and P3H1 secreted into the ECM. CRTAP is thought to function as a helper protein within the complex. It is highly homologous to the N-terminal end of P3H1.[105] P3H1 is encoded by *LEPRE1* (leucine- and proline-enriched proteoglycan 1), and is the only member of the complex to possess a KDEL ER-retention/retrieval signal, although it also has a secreted isoform called leprecan.[106] CRTAP and P3H1 provide mutual stabilization for each other in the complex, since the levels of both proteins are severely reduced in cells that have null mutations in either gene, even though mRNA levels of the unaffected gene are normal.[107]

The members of the 3-hydroxylation complex were implicated in recessive OI when four independent research approaches came together: (1) the Bachinger lab isolated the elusive P3H1, and found it occurred as a complex with CRTAP and CyPB;[108] (2) the Marini lab hypothesized that recessive OI with overmodified collagen was caused by defects in protein(s) that interacted with type I collagen and took a candidate gene approach to identifying those proteins;[45] (3) *Crtap*-null mice were characterized with a recessive osteo- and chondrodysplasia with rhizomelia and osteopenia;[10] and (4) the Glorieux group had described a First Nations pedigree with recessive OI and rhizomelia that mapped to chromosome 3p22 (designed type VII OI before a cause was identified).[109] Subsequently, type VII OI has been shown to be caused by homozygous mutations in *CRTAP*, predominantly resulting in null alleles with absent or minimal transcript levels. This abrogates Pro986 3-hydroxylation and, unexpectedly, resulted in overmodification of the helix with levels of hydroxylysine and 4-hydroxyproline similar to those seen in collagen with structural mutations at the carboxyl-end of the helix. The collagen overmodification implies that helix folding is delayed. Furthermore, in cultured dermal fibroblasts from patients, the amount of collagen incorporated into matrix is about 10% of normal levels, suggesting that the OI phenotype is the result of an abnormal ECM, as well as an overmodification of type I collagen.

Type VIII OI is caused by mutations in *LEPRE1*, which encodes P3H1, the enzymatic component of the 3-hydroxylation complex.[11,46] Biochemically, type VIII is similar to type VII in that Pro986 3-hydroxylation is reduced, collagen type I is overmodified and there is a reduced amount of collagen secreted into the matrix. A single founder mutation (c.1080 + 1G > T) occurring in 0.4% of African-Americans and 1.5% of West Africans accounts for almost half of OI type VIII cases reported.[71]

CyPB is the third component of the 3-hydroxylation complex. It is an ER-localized cyclophilin, encoded by the peptidyl-prolyl *cis–trans* isomerase B (*PPIB*) gene. The conversion of *cis*-proline to the *trans*-isomer is rate limiting during collagen folding, and it was thought that CyPB was the unique isomerase acting on collagen. Some patients with *PPIB* mutations have Pro986 3-hydroxylation reduced to about 30%, a lesser reduction than the 3%–5% residual hydroxylation in those with mutations in *CRTAP* or *LEPRE1*.[47,48] Again, the collagen is overmodified, potentially as a result of lack of CyPB *cis–trans* isomerase activity. However, a *PPIB* homozygous start codon mutation resulted in total absence of CyPB, but normal Pro986 3-hydroxylation and collagen folding, suggesting the potential for redundant

isomerase activity involved in collagen prolyl *cis–trans* conversion (see *Ppib* in Section 5.2).[12]

4.4 Defects in Collagen Processing and Crosslinking (Types X–XII OI)

Molecular chaperones resident in the ER are involved at many stages of type I procollagen synthesis, folding, and export. These chaperones not only ensure that alpha chains are properly modified and folded, they also function to prevent their aggregation prior to attaining their final tertiary structure. Many collagen-interacting chaperones are multifunctional and have different domains with distinct roles, which may be related to or independent of their chaperone function. Certain chaperones, such as HSP47, are collagen-specific and others, such as BiP, GRP94, and PDI, have a wider range of targets.

HSP47, encoded by *SERPINH1*, was an excellent candidate gene for recessive OI because of its collagen-specific chaperone activity. The first HSP47 defect was characterized in dachshunds, with the candidate gene identified via homozygosity mapping. Three independent homozygous mutations resulting in amino acid substitutions have now been reported in patients with type X OI.[13,54,55] In vitro, HSP47 preferentially binds fully folded triple helical collagen molecules, consistent with a role in inhibiting helical denaturation and preventing procollagen aggregation during transport through the secretory pathway.[110] Although mutant HSP47 was subject to almost complete degradation, type I procollagen helical modification was normal. However, the increased susceptibility of the helical region of secreted collagen to protease digestion suggested that the helix was not correctly folded.[13] Furthermore, the presence of mutant HSP47 led to decreased FKBP65 protein and aggregation of procollagen in ER-adjacent compartments, possibly autophagosomes.[54] The same investigation demonstrated that absence of FKBP65 does not affect HSP47 levels, but instead results in HSP47 mislocalization to these ER-adjacent compartments, suggesting that both chaperones cooperate in procollagen trafficking from the ER to Golgi compartments.

Defects in *FKBP10*, encoding FKBP65, encompass a spectrum of disorders that includes type XI OI, Bruck syndrome and Kuskokwim Syndrome. Deficiency of *FKBP10* was identified as the cause of recessive type IX OI in a group of consanguineous Turkish families.[14] Subsequently, Bruck syndrome I (BRKS1, OMIM #259450), a recessive disorder of severe OI with congenital contractures, was also shown to result from *FKBP10* mutations.[50] Kuskokwim syndrome, a congenital contracture syndrome with minimal skeletal manifestations that occurs among the Yup'ik Eskimos of Alaska, is caused by a founder mutation resulting in an in-frame deletion of a conserved tyrosine in the third PPIase domain of FKBP65.[53] Cells with mutant FKBP65 have normal collagen Pro986 3-hydroxylation and normal helical post-translational modification.

Bruck syndrome II (OMIM #609220) is caused by mutations in *PLOD2*, which encodes lysyl hydroxylase 2 (LH2), the enzyme responsible for hydroxylation of collagen telopeptide lysine.[111–113] Both LH2 and FKBP65 deficiencies result in severely reduced hydroxylation of the type I collagen telopeptidyl lysine residues, required for crosslinking in the ECM. This striking identical biochemical outcome is due to the requirement of FKBP65 PPIase activity for the formation of enzymatically active LH2 dimers from its inactive monomeric form.[114]

OI due to homozygous or compound heterozygous mutations in *BMP1*, encoding the metalloproteases bone morphogenetic protein-1 (BMP1) and its longer isoform mammalian Tolloid (mTLD), is classified as type XII OI. BMP1/mTLD functions as the types I–III procollagen C-propeptidase and the N-propeptidase of type V procollagen, but also participates in proteolytic trimming of other ECM components including prolysyl oxidase, the proteoglycans prodecorin, and probiglycan, and dentin matrix protein 1 (DMP1), regulators of collagen fibrillogenesis and mineralization.[115–117] Some, but not all, patients with BMP1 deficiency have exhibited "high bone mass OI", characterized by high areal bone mineral density and hypermineralization of bone by qBEI, similar to findings resulting from dominant mutations in the type I procollagen C-propeptide cleavage site.[15,56–58,60,86,118] Recessive *BMP1* mutations cause a more severe phenotype than C-propeptide cleavage site defects, presumably due to failure of BMP to process its noncollagenous substrates. In both types of processing defects, incorporation of pC-collagen into ECM results in irregularly shaped, larger diameter fibrils, and may provide an increased number of sites for mineral nucleation in bone matrix.[57,86]

4.5 Defects in Osteoblast Differentiation and Function (Types XIII–XVIII OI)

The six OI-causative genes grouped together functionally for osteoblast defects were identified in patients with moderate to severe clinical and radiographic OI. Their protein products do not directly interact with type I collagen for functions in modification or assembly, although they all impact the amount of type I collagen secreted from the cell. Furthermore, functional studies of mutations in three of these genes (*TMEM38B*, *SPARC*, and *MBTPS2*) showed that they affect collagen modification, in addition to their general impact on cellular differentiation. Much remains to be learned about the functional intersection of these osteoblast pathways and proteins that interact directly with collagen, and how they lead to a common bone dysplasia.

For *SP7/OSTERIX* (*OSX*), a single child with a homozygous deletion (c.1052delA) has been identified.[16] The proband has moderately severe OI (type XIII OI), with frequent fractures (6–7 per year), bowing of upper and lower limbs, wormian bones, midface hypoplasia, short stature and generalized osteoporosis, as well as atypical features including mild asymmetry of the face, micrognathia, and hyperextensibility of small joints. *SP7/OSX*, a transcription factor expressed in cortical and trabecular osteoblasts and to a lesser extent in prehypertrophic chondrocytes of the growth plate, has an essential role in regulating the differentiation of preosteoblasts to osteoblasts downstream of *RUNX2*. No functional studies of the patient's mutation were performed. However, *Osx*-null mice demonstrate defective osteoblast differentiation, with reduced expression of *Col1a1*, *Bsp*, and *Osteocalcin*.[119]

Type XIV OI was originally reported as a founder mutation in Bedouin families from Israel and Saudi Arabia.[17,64] A deletion of exon 4 and surrounding intronic sequence leads to a moderately severe bone phenotype. Five independent mutant alleles have now been identified, each resulting in the introduction of PTCs, nonsense mediated decay of transcripts, and total absence of its protein product.[17,61–64] *TMEM38B* encodes the integral ER membrane K^+ channel TRIC-B, first proposed to support IP_3R-mediated calcium release from intracellular stores.[120] Further investigation into the mechanism of TRIC-B deficiency has demonstrated a role for TRIC-B in modulating the kinetics of ER calcium depletion and recovery.[61] More relevant to the collagen-related paradigm for OI, disturbed Ca^{2+} flux results in chronic elevation of the PERK pathway of ER stress with increased levels of intracellular BiP. Furthermore, disturbed Ca^{2+} flux dysregulates type I collagen biosynthesis by impacting the function of multiple collagen-specific chaperone and modifying enzymes. Reduced helical lysine hydroxylation, delayed chain assembly, and intracellular retention of misfolded collagen species all contribute to the severely reduced quantities of procollagen secreted by proband osteoblasts and to matrix insufficiency.[61]

For type XV OI, a total of 22 unique disease-causing variants in the *WNT1* coding region have been reported. Unusual for an OI type, heterozygous *WNT1* mutations lead to osteoporosis, while homozygosity for *WNT1* mutations cause severe OI.[18] WNTs (wingless-type MMTV integration site family members) are secreted signaling proteins, many of which are known to be key regulators of bone mass.[121–123] Altered WNT1 is unable to activate β-catenin via its osteoblast cell surface receptor, low-density lipoprotein receptor-related protein 5 (LRP5), to stimulate bone formation. Interestingly, deficiency of *LRP5* causes juvenile osteoporosis, resembling moderately severe (type IV) OI in its skeletal features.[124–126] Learning and developmental delays due to brain malformations have only been observed in a few patients.

In two unrelated probands with moderately severe OI (type XVII OI), homozygous variants in *SPARC* (p.R166H and p. Q263L) have been identified.[20] Secreted protein, acidic, and cysteine-rich (SPARC), also known as osteonectin, is a collagen-binding glycoprotein involved in ECM synthesis and calcification. SPARC residues Arg166 and Glu263 are essential for SPARC binding to type I collagen.[127] Studies using proband fibroblasts revealed delayed type I collagen electrophoretic mobility, consistent with overmodification, and decreased collagen secretion, raising the possibility that SPARC plays a role as an intracellular collagen chaperone. Furthermore, BMDD of proband trabecular bone by qBEI was increased, consistent with the hypermineralization of classical OI and suggesting a role for SPARC in bone mineralization.

The RIP pathway, well-studied for its role in cholesterol metabolism, had been implicated in bone formation in mice and zebrafish mutants.[128,129] Defects in two components of this pathway have now been identified in OI patients. First, a homozygous genomic deletion of *CREB3L1*, encoding OASIS (*O*ld astrocyte specifically induced substance) was identified in two siblings, now classified as type XVI OI (OMIM #616229).[19] OASIS, like ATF6, is a member of the ATF/CREB basic-leucine zipper (bZIP) DNA-binding protein family and functions as an ER-stress transducer. OASIS is highly expressed in osteoblasts, where it is processed by RIP in response to ER stress.[130] The N-terminal cytosolic portion of OASIS is subsequently translocated to the nucleus, where it binds to the osteoblast-specific unfolded protein response element (UPRE) of *COL1A1* and other bone ECM-associated genes to upregulate their expression. Since decreased expression of structurally normal type I collagen is typically associated with mild OI (type I), the severity of the OI phenotype in these probands suggested critical roles for the osteoblast-specific stress response and the process of RIP in bone development.

Also in the RIP pathway, the first X-linked recessive form of OI (type XVIII OI) was shown to be caused by missense mutations in *MBTPS2*, which encodes site-2 protease (S2P).[21] S2P is an integral Golgi membrane protein that, together with S1P, sequentially cleaves regulatory substrates transported from the ER membrane in response to ER-stress or sterol restriction by the process of RIP.[131,132] In two independent pedigrees with moderate to severe OI and an X-linked inheritance pattern, linkage analysis and next-generation sequencing (NGS) identified novel *MBTPS2* missense mutations that cosegregated with the OI phenotype.[21] In both pedigrees, the resulting substitutions (p.N459S and p.L505F) occur in or adjacent to the metal ion coordinating site required for enzymatic activity. Mutant S2P is stable but processing

of the RIP substrates ATF6, OASIS, and SREBP are impaired, thereby reducing expression of several genes required for osteoblast differentiation and ECM synthesis. Type XVIII OI was also connected to the collagen-related paradigm of OI by demonstration of abnormal modification in collagen extracted from bone tissue. Collagen crosslinks involving the K87 residue were about 50% hydroxylated in both α1(I) and α2(I), versus near complete hydroxylation in normal bone. The K87 residue is modified by LH1, which was previously unknown to be affected by the RIP pathway. Interestingly, multiple other substitutions in *MBTPS2* had previously been associated with the dermatological conditions IFAP (ichthyosis follicularis, atrichia, and photophobia) and keratosis follicularis spinulosa decalvans.[133–135] X-OI patients do not have symptoms of IFAP and conversely, IFAP patients do not have symptoms of OI. Although the mechanism(s) by which different mutations in *MBTPS2* cause different disorders remains to be delineated, one major distinction was found to reside in the urinary bone crosslink profile, with elevated LP/HP ratios in X-OI but not IFAP patients.

5 ANIMAL MODELS

Several mouse models have been engineered to aid in the analysis of the bone phenotypes and disease mechanisms of OI, and to test the efficacy of potential therapeutic treatments. Most of the models recapitulate type I collagen defects, although with the recent discovery of genes involved in the recessive OI forms, knockout models for these genes are now of increasing interest.

5.1 Dominant Type I Collagen Murine Models

The first OI mouse model, designated Mov13, was generated in 1983 by the random integration of Moloney murine leukemia retrovirus (M-MuLV) DNA into the first intron of the mouse *Col1a1* gene.[136] Mice homozygous for Mov13 ($Mov13^{-/-}$) are embryonic lethal at about E13 due to absence of *Col1a1* mRNA transcription by osteoblasts. Heterozygous mice ($Mov13^{+/-}$) synthesize normal proα1(I) at about half the normal level, have a much milder phenotype with normal life expectancy and fertility, and represent a model for type I OI. However, dentin forming odontoblasts appear to synthesize normal levels of type I collagen throughout development and postnatal life.[137] These findings may shed light on the cell-specific transcriptional controls involved in type I collagen expression.[138,139]

The osteogenesis imperfecta murine (*oim*) mouse has a naturally occurring single base pair deletion in the *Col1a2* gene, at position 3983 (c.3983delG), which shifts the reading frame and alters the last 48 amino acids of the protein, containing the chain alignment region.[140] Homozygous mice failed to produce any functional proα2(I) protein, even though the mRNA was transcribed and stable. As a result, these mice synthesized only α1(I) homotrimers, and had a moderate to severe type III OI-like phenotype characterized by skeletal fractures, progressive limb deformities, osteopenia, and reduced bone mineral density. Architecturally, bone had thinner cortices, and reduced trabecular volume. Mineral composition was also altered, with the size and alignment of crystals being affected by the lack of α2(I) chains in the collagen.[141] Given that in humans, proα2(I) null mutations lead to EDS and not OI, the *oim* bone phenotype is presumptively the consequence of mutant proα2(I) chains in the ER, which cannot incorporate into heterotrimers.

In the last decade, three OI mouse models were generated using knockin or random mutagenesis technologies, each containing a classical OI collagen mutation equivalent to mutations identified in patients. The first OI knock-in mouse was the Brtl mouse, into which a reproduction of a *COL1A1* mutation causing a classical glycine substitution at position 349 (c.1546G > T; Gly349Cys) was introduced.[142] This mutation had been identified in a child with type IV OI. $Brtl^{+/-}$ mice have two discrete phenotypes: 30% of $Brtl^{+/-}$ mice are perinatal lethal, 70% are nonlethal with normal fertility and lifespan. Heterozygous Brtl mice displayed a moderately severe OI phenotype, including small size throughout the lifespan, flared ribcage, fragile undermineralized bone with bulbous metaphyses, and trabecular disorganization. The phenotypic divergence was independent of mRNA or protein expression levels, and has similarities to the wide, discrete phenotypic variability seen predominantly with some *COL1A1* mutations. However, variable expression of OI may involve cytoskeletal components, since compromised formation and organization of microtubule and intermediate filaments has been associated with impaired cell signaling and cellular trafficking in lethal mice.[143]

On microCT analysis, Brtl femora have reduced cortical thickness, cross-sectional area, and bending moment of inertia; additionally, they have decreased postyield displacement, indicative of brittle bone tissue. Brtl femora show reduced yield and failure loads at 2 months, but are comparable to wild-type mice at 6 months of age despite persistence of thin bone geometry.[144] These findings suggest that Brtl bone has a postpubertal adaptation involving changes in matrix material properties rather than improved bone geometry, as seen in Mov13 mice. Improved elastic modulus and predicted ultimate strength of 6-month Brtl femora support the interpretation of Brtl adaptation by altered bone material properties.

Histomorphometry of Brtl femora demonstrated uncoupling of osteoblasts and osteoclasts, with normal

osteoblast surface but a decreased matrix mineralization rate in Brtl, along with increased osteoclast number, plus larger, and more intensely TRAP-staining cells.[145] The increased osteoclast number is independent of the RANKL/OPG ratio, indicating that an as-yet-unidentified factor is crucial for the high-osteoclast number and high turnover of Brtl bone. This cellular imbalance leads to the Brtl reduced net bone formation rate.

Further insight into the mechanism of OI in bone tissue has been obtained from proteomic and cell differentiation analyses. Comparison of the calvarial bone of lethal and nonlethal Brtl mice using microarray, real-time RT-PCR, and two-dimensional bone proteomic profiles, revealed that expression of the ER stress-related protein CHOP was increased in lethal mice, whereas expression of the chaperone αB crystalline was increased in nonlethal mice, supporting a role for osteoblast cell stress in bone pathology.[146] Brtl bone marrow stem cells showed a reduced capacity to differentiate toward osteoblasts, but an increased ability to differentiate toward the adipogenic lineage.[147] The increased levels of BECN1, ATG7, and LC3-II in differentiating mutant MSCs led to the hypothesis that intracellular retention of mutant collagens triggers autophagy, resulting in transdifferentiation of preosteoblasts into preadipocytes.

The Brtl mouse is a useful tool in assessing the effectiveness of both current and potential OI treatments, including modeling the effects of bisphosphonate treatment regimens, and determining the efficacy of transplanting normal bone marrow cells into mutant bone. Treatment of Brtl mice with the bisphosphonate alendronate showed that although bone geometry and load to fracture were improved, this was associated with numerous adverse effects.[148] Alendronate treatment leads to a striking retention of mineralized cartilage, decreased matrix synthesis and material strength, and altered osteoblast morphology from cuboidal cells to flat lining cells. The murine data indicate the importance of limiting the duration of bisphosphonate therapy in children, to obtain increased bone volume while minimizing the detrimental effects on bone cells and material properties. Brtl is also being utilized to model the effects of antisclerostin antibody on OI bone. This agent has an anabolic mechanism involving release of sclerostin inhibition of the Wnt pathway. Antisclerostin antibody increases Brtl bone formation rate, mineral apposition rate, and bone volume.[149–151] Cortical thickness is increased, with no detrimental effect on tissue mineralization, requiring increased force to break Brtl femora. Finally, the brittleness of Brtl bone was improved, holding considerable promise for treatment of OI and supporting initiation of clinical pilot studies.

The second knock-in mouse modeled a *COL1A2* glycine substitution mutation identified in 64 affected individuals of a large Old Order Amish (OOA) kindred. The OI phenotype in this kindred ranged from Sillence type I to IV OI, due to a G > T transversion at position 2098 in *COL1A2* (c.2098G > T; Gly610Cys).[152] Generally, $OOA^{+/-}$ mice were smaller, had reduced bone mineral density and increased susceptibility to bone fractures. The detection of increased cortical volumetric tissue mineral density (vTMD) on microCT analysis accurately reproduces the paradoxical increase in total mineralization of OI patient bone samples. When the mutation was crossed into four different mouse strains, bone geometry and mechanical properties (failure to load and postyield displacement) varied among the F1 hybrids, indicating that these characteristics may be modulated by noncollagenous genetic factors. Quantitative trait loci analyses to link phenotypic variability to genetic differences could lead to the identification of such modifiers.

The third murine model, $Aga2^{+/-}$ (abnormal gait 2), was generated by ENU mutagenesis and has a mutation in the C-propeptide coding region of *Col1a1* (c.4216-2T > A).[153] The mutation introduces a novel splice acceptor 16 bp upstream of the normal exon 51 splice acceptor site. At the protein level, this caused a frameshift of the last 48 amino acids and the addition of a further 90 amino acids beyond the original stop codon. The frameshift resulted in the removal of a highly conserved cysteine residue involved in intrachain disulfide bond formation and adversely affects the sequence involved in the selection of monomer chains during C-terminal trimerization. The severity of the $Aga2^{+/-}$ phenotype recapitulates the human type II–III OI phenotype. Deformities of the hind limb were observed in 5-week-old mice, and a large number of newborn mice were perinatal lethal. In addition to severe bone deformities, the mice had multiple fractures, osteoporosis, disorganized trabeculae, and disrupted matrix organization, associated with increased bone turnover. In cultured dermal fibroblasts, abnormal proα1(I) chains accumulated within the ER, and induced an UPR response as demonstrated by an increase in BiP (*Hspa5*), Hsp47 (*SerpinF1*), and Chop (*Gadd153*) expression. Apoptosis of osteoblasts was also increased, potentially mediated by the increase in *Gadd153* expression.

Studies of $Ifitm5^{-/-}$ mice revealed only minor effects on bone, including reduced bone length and bowing of long bones in newborns, that resolved with age.[154] Knock-in mice expressing the type V OI-causing mutation (*Ifitm5* c.-14C > T) are embryonic lethal, and have severely undermineralized long bones with unresorbed-cartilage anlage.[155] A transgenic model with bone-specific expression of the *Ifitm5* type V OI-causing mutation two- to fivefold higher than endogenous *Ifitm5* exhibited perinatal lethality with severe defects of long bones and ribs.[156] Histology of transgenic embryos showed persistence of a cartilage-like matrix throughout bone tissue. Although most hypotheses posit an activating role for the type V causative mutation, expression of osteoblast-specific genes *Sp7*,

Col1a1, *Bsp*, and *Ocn* were reduced in transgenic calvarial bone tissue. A surviving model, that recapitulates the classic triad of type V OI clinical features, including dense metaphyseal lines and mineralization of the interosseous membrane, remains to be generated.

5.2 Recessive Murine Models

A *Serpinf1* knockout mouse was originally generated to study the role of PEDF in the inhibition of angiogenesis. In addition to vascular and epithelial abnormalities, loss of PEDF in mice produces skeletal features, which resemble the main defects seen in type VI OI patients.[157] Histomorphometric analyses revealed normal numbers of osteoblasts and osteoclasts in PEDF-deficient mice with a normal rate of bone formation. However, *Pedf*$^{-/-}$ mice exhibited significant increases in osteoid thickness and maturation time, consistent with a defect in the mineralization process. In both mouse femora and primary osteoblast cultures, ECM demonstrated an increased mineral to matrix ratio, and was associated with decreased expression of genes involved in the mineralization process, including *Npp1*, *Dmp1*, *Phex*, and *Bsp*.

The knock-out mouse models for genes involved in recessive OI defects in the 3-hydroxylation complex proteins have generally milder phenotypes than their human counterparts, but do recapitulate the biochemical features of their respective OI types. These mouse models will be indispensable for investigating the relative contributions of lack of collagen 3-hydroxylation versus absence of the hydroxylation complex, and the overlap in recessive and dominant OI mechanisms at the bone level.

The *Crtap* knockout mouse was generated by homologous recombination before the first cases of type VII OI were identified; it displays a complete absence of *Crtap* transcripts.[10] Procollagen secreted in culture, as well as bone and dermal tissue derived collagen, completely lacks Pro986 3-hydroxylation, but has full helical overmodification. Mice had a moderate nonlethal skeletal phenotype, with pre- and postnatal growth deficiency, rhizomelia, and progressive kyphoscoliosis, as well as extraskeletal abnormalities in lung and kidney, with increased alveolar spacing and segmental glomerulosclerosis, respectively.[10,158] In the skeleton, there is significant osteoporosis with reduction of bone volume, trabecular number, and thickness, as well as reduced bone formation rate. The bone matrix of *Crtap*$^{-/-}$ mice has a significant increase in mean calcium concentration, and reduction in mineralization heterogeneity.[159]

The murine null model for type VIII OI with P3H1 deficiency (*Lepre1*$^{-/-}$) confirmed the importance of the complex for bone and cartilage structure, although the overall phenotype is milder than in patients.[160] As expected, Pro986 3-hydroxylation was absent in *Lepre1*$^{-/-}$ mice, with evidence of overmodification and delayed collagen secretion. *Lepre1*$^{-/-}$ mice survive to adulthood and exhibit a moderate OI phenotype with growth deficiency, progressive kyphoscoliosis, delayed parietal bone ossification, rhizomelia, reduced femoral strength, and cortical thickness. Trabecular thickness is also reduced, but not to the extent of the extremely thin trabeculae seen in type VIII OI patients, reflecting the less severe phenotype in P3H1-deficient mice than children.[161,162] The *Lepre1*-null mice also do not model the patchy osteoid seen in type VIII children. P3H1 is also expressed in chondrocytes and the growth plate revealed severe disorganization of the hypertrophic zone. Similar to what is observed in *Crtap*-null mice, qBEI analyses revealed hypermineraliztion of P3H1-deficient murine bone, except *Lepre1*-null mice retain residual mineralized cartilage tissue within their trabecular bone, which may contribute to lower bone quality in these mice.[161] Finally, neither the *Crtap*- nor *Lepre1*-null mice have high-bone turnover. Instead they have reduced bone formation and normal to low-osteoblast and osteoclast indices, suggestive of an osteoblast defect.[10,161]

The mouse model for deficiency of the third component of the complex, *Ppib* (CyPB), exhibits a 30%–40% perinatal lethality rate, growth deficiency, kyphosis, and severe osteopenia, but does not have rhizomelia.[163] The structural and mechanical properties of *Ppib*$^{-/-}$ femora are consistent with reduced trabecular and cortical bone formation, resulting in weaker, more brittle bones. In *Ppib*$^{-/-}$ fibroblast and osteoblast cell cultures, collagen was shown to fold more slowly in the absence of CyPB, supporting its proposed rate-limiting role in folding. However, treatment of CyPB-deficient cells with cyclosporine A caused a further delay in folding, indicating the likely existence of another collagen PPIase. Collagen synthesized by *Ppib*$^{-/-}$ fibroblasts and osteoblasts is overmodified, with nearly complete absence of P986 3-hydroxylation. Furthermore, this study extended the reported role of CyPB in collagen lysyl hydroxylase (LH1) activity by demonstrating site-specific alterations in hydroxylation of helical lysine residues involved in crosslinking.

Tricb (*Tmem38b*) knock-out mice die immediately after birth due to respiratory failure caused by insufficient surfactant.[164] In both humans and mice, absence of TRICB inhibits IP_3R-mediated Ca^{2+} release from intracellular stores. However, while human osteoblasts demonstrate normal resting levels of ER Ca^{2+}, data from the *Tricb*-deficient mouse has been interpreted as ER Ca^{2+} store overload in osteoblasts.[61,165] Analysis of *Tricb*-deficient embryonic bones have shown impaired mineralization of the skull, ribs, and femora, consistent with reduced collagen content. Both osteoblast and osteoclast numbers were reduced in femora, with decreased levels of *Ocn*, *Sparc*, calcitonin receptor (*Ctr*), and *Ctsk*. Furthermore,

osteoblasts were noted to have dilated rERs containing dense collagen deposits. The femoral growth plate was normal. It was hypothesized that disruption of collagen maturation or intracellular trafficking in *Tricb*-deficient mice leads to induction of ER stress and the UPR in osteoblasts.[165]

Originally described for its neurological features, the *Wnt1* knock-out mouse has severe abnormalities in brain development, with a skeletal phenotype that has only recently been appreciated.[166,167] *Wnt1*$^{sw/sw}$ mice exhibit spontaneous fractures and severe osteopenia. In contrast to collagen-related forms of OI, mechanical testing revealed that postyield displacement of *Wnt1*$^{sw/sw}$ is increased, indicating less brittle bones than in wild-type mice. Reduced expression of osteoblast markers *Col1a1*, *Alpl*, and *Ocn*, and normal osteoclast markers reflected the decreased osteoblast activity that results in bones with lower collagen and mineral content.

The initial characterization of the *Oasis* (*Creb3l1*) knock-out mouse created considerable interest in the OI research community as the phenotypic features made it an ideal candidate gene for unidentified cases of recessive OI. OASIS-deficient mice exhibit severe osteopenia and growth deficiency involving a decrease in type I collagen expression, synthesis, and matrix deposition.[168] Although, bone-specific reintroduction of *Oasis* expression rescued the osteopenic phenotype, it was not sufficient to reverse growth deficiency in these mice. Rather, growth deficiency in *Oasis*$^{-/-}$ mice was reversed by growth hormone treatment, leading to the speculation that OASIS regulates skeletal development by osteoblast-dependent and -independent mechanisms.[169]

6 DIAGNOSTIC ASPECTS

The diagnosis of OI and the designation of particular types of OI involve clinical, radiographic, genetic, and molecular criteria.[7] Clinical signs include osteopenia, skeletal deformity, such as bowing and undertubulation, fractures, short stature, scleral hue, DI, hearing loss, and relative macrocephaly. Differential diagnostic questions arise in successive developmental timeframes, as to which types of OI should be under consideration versus other skeletal conditions. The prenatal diagnosis of OI by ultrasound can be accomplished for the more severe dominant (II and III) and recessive (VII, VIII, IX, X) types as early as the 16th week of gestation.[6] Typical ultrasound findings include low echogenicity of the skeleton because of poor mineralization, and deformity of long bones, including shortening, bowing, and fractures. Prenatally, severe OI can be difficult to distinguish from some of the chondrodystrophies, such as thanatophoric dysplasia, campomelic dysplasia, and achondrogenesis type I. If the diagnosis is first made by ultrasound, amniocytes can be used for molecular, but not biochemical testing because the high production of α1(I) chains would result in a false positive biochemical test. If prenatal testing is prompted by the risk of recurrence, chorionic villi are available early in gestation and can be used for both molecular and biochemical testing.[170]

In the newborn period, many moderately severe cases will be diagnosable, as well as the severe types. Severely affected infants with dominant OI will often have a large poorly mineralized cranium, blue sclerae, a thorax that narrows at the apex, fractured limbs, and lower extremities held abducted in the frog-leg position. Radiographically, typical findings include wormian bone, a rib cage with beads of callus on multiple ribs, undertubulated long bone, and crumpled fractures in various stages of healing. Severe infants with recessive OI will typically have a normal to small cranium with poor mineralization, and white sclerae. However, the skeletal appearance of recessive OI overlaps with the severe dominant types. Biochemical testing will not distinguish between severe autosomal dominant OI and recessive OI types VII and VIII, since the biochemical test will show full overmodification in all cases of types VII and VIII OI, similar to severe dominant OI caused by mutations near the carboxyl end of the collagen helix.[11,45] Suspicion for recessive forms is also heightened by recurrence in a family, or a consanguineous genetic background, as well as clinical features. However, collagen defects are sufficiently more frequent than recessive forms that even in the cases of a severe infant of West African descent, one must think first of a collagen mutation, and second of the *LEPRE1* founder allele. If dermal fibroblasts are available, a quick screening for transcript levels of the genes causing recessive types in the same phenotype range can confirm the most productive screening approach. If only DNA is available, initial sequencing for dominant mutations in *COL1A1*, *COL1A2*, and *IFITM5* is the most direct route, with subsequent sequencing of a recessive gene panel if the dominant panel reveals no abnormalities.

Moderately severe OI cases include dominant types IV (predominantly) and V OI, and recessive type XI OI (*FKBP10* mutations) and some cases of type IX (*PPIB* mutations). These recessive types are rare and almost all moderately severe cases will have collagen defects. Radiographs will show classic signs of OI but will not distinguish autosomal dominant and recessive types. Type V OI, caused by a recurrent 5′ *IFITM5* mutation, does have distinguishing features of dense metaphyseal bands, ossification of the interosseous membrane and hypertrophic callus, as well as a mesh-like appearance on bone histology.[8]

Recurrences of affected offspring to unaffected parents may be due to either parental mosaicism for a dominant mutation or to a recessive condition. Estimates vary from 5% to 10% for the proportion of first affected

children with a collagen mutation who inherited a mutant allele from a mosaic parent.[171] Mosaic carriers may have soft signs, such as blue sclerae, mildly short stature, osteopenia, DI, history of fracture in childhood, or they may be totally asymptomatic. Once the collagen mutation in the child is known, the mosaic parent can be identified by careful analysis of leukocyte DNA for a low percentage of mutant sequence. Recessive OI comprises a similar proportion of total OI cases as mosaics with dominant disease. Parental carriers of recessive OI have come predominantly from cultures, which encourage consanguineous marriages, from geographic isolates, or founder mutations. Careful studies of carrier parents for recessive OI have not been published.

Some OI (types I and IV) may be misdiagnosed as child abuse.[172] Decreased bone density, wormian bones, DI, and bowing of long bones can be helpful in the differential diagnosis. Blue sclerae are not as helpful in infancy, when many normal children have a blue scleral tinge. Children with OI generally do not have bruising at the site of the fractures. Rapid molecular diagnosis can be definitive when the distinction is subtle.

7 TREATMENT

The goal of OI treatment is to maximize the motor function and independence of the patient. This includes, but is not purely focused on, the reduction of fractures, since often the least active children can have the fewest fractures. Instead, the salient questions should focus on the degree of trauma needed to cause a fracture and the independence of the patient in their home, school, and community.

Rehabilitation medicine and physical therapy are the foundation of a successful intervention for OI patients and cannot be replaced by drug therapy. Early intervention for infants with severe OI focuses on positioning of head and spine to prevent torticollis and reduce contractures from abduction. Although motor skills will lag behind unaffected children, with an individualized program of muscle strengthening and aerobic conditioning (including water therapy and swimming), children can be prepared for sitting, standing, and in most cases, protected ambulation. Functional tests, such as the BAMF (brief assessment of motor function) have been validated for OI and can be used to track progress.[173] At the milder end of the scale, children with types I and IV OI who participated in a low-resistance physical training program showed increased muscle strength and improved peak oxygen consumption, but required a regular program to maintain these gains.[174] Prevention of scoliosis is a major goal of physical rehabilitation for moderate and severe OI patients. OI children with hyperlaxity may develop early and aggressive scoliosis from laxity of paraspinal ligaments, as well as asymmetric vertebral compressions. Conventional bracing is not applicable or successful in OI. Strengthening back muscles and ligaments through suspension exercises has shown early promise as a supplement to pharmacological therapy.

Orthopedic surgery is an integral part of the management of OI.[175] To preserve long bone function, fractures should not be allowed to heal without reduction and should have the shortest immobilization time consistent with adequate bridging, to avoid worsening of osteoporosis by disuse. Intramedullary-rod placement will be considered in moderate and severe cases for functional purposes, such as bearing weight and assisted ambulation, after children are ready to stand. Rods may also be useful to interrupt a fracture cycle in the same long bone, holding ends in alignment during healing. Considerations, such as the child's developmental progress and whether to intervene electively when bowing exceeds 40°, or when valgus/varus deformities interfere with gait, are parts of the presurgical evaluation. Most moderate and severely affected children will have improved neuromuscular development and meet milestones at a more normal rate with rodding of lower extremity long bones before age 3.5 years. Telescoping rods, such as the Sheffield and the newer Fassier–Duval, which allows for percutaneous placement, are used in children with good growth potential; rates of rod migration are similar in both systems.[176,177] Straight pins and Rush rods have a place in severe OI where lower growth rates are expected; these rods unload the bones less than the telescoping rods and provoke less diaphyseal atrophy.[178] The progressive scoliosis of OI does not respond to conservative management with bracing. To maximize preservation of lung function and control pain, correction should be done before the curve reaches 60°.[179] European teams have pioneered use of halo traction followed by spine stabilization with good success.[180]

Growth deficiency is one of the most consistent features of OI, varying from final stature in the prepubertal range to shorter than same-gender siblings in mild cases. Only a modest portion of this growth deficiency can be attributed to radiographic popcorn in epiphyses.[181] Severely affected infants fall below growth curves by 1-year of age, and several years of growth plateau during preschool years leads to a further lag in growth.[182] In many cases of recessive OI with deficiency of components of the 3-hydroxylation complex, which are normally expressed in both chondrocytes and osteoblasts, the growth deficiency is extreme, and final stature can be that of an average 3-year old.[11,12,46] About half of children with type IV OI and nearly all children with type I OI treated with standard doses of recombinant growth hormone had a substantial increase in linear growth rate.[183] Some type IV OI children have even attained statures in the normal range. Type IV OI children who respond

with increased linear growth also have improved bone volume on histomorphometry, increased bone density, and decreased fracture rates, while bone age is not advanced beyond chronological age.

The pulmonary and cardiovascular systems are the major proximal cause of morbidity and mortality in OI.[184] Severely affected children will have recurrent pneumonias, sometimes fatal. Older children and adults often have restrictive lung disease, due to kyphoscoliosis, with decreased pulmonary function consistently related to scoliosis greater than 60°, and chest wall deformity.[179] Even pediatric patients without scoliosis show progressive restrictive pulmonary disease, supporting a primary intrinsic abnormality in the lung parenchyma.[185] Children with OI should have periodic formal respiratory evaluations, including spirometry, since they may not have symptoms with early or moderate lung dysfunction, which should be treated with standard medications. In adults, this progresses to right-sided heart failure (*cor pulmonale*). In addition to *cor pulmonale*, cardiac structural findings have been reported in children and adults with OI.[185] Among adults with otherwise asymptomatic OI, 95% were found to have aortic root dilatation and valvular regurgitation, with tricuspid and mitral regurgitation (with or without aortic regurgitation) accounting for 60% of cases.[186,187]

Hearing loss is a common secondary feature of dominant OI and is a progressive condition. About one-third of affected children have mild hearing loss that does not interfere with daily functioning, and even in childhood, 5% will have loss of 20 dB or greater.[188] The loss most commonly becomes functionally significant by the 3rd decade, with moderate or severe combined sensorineural and conductive loss in about 7% of affected individuals. Formal hearing assessments should begin in childhood and be repeated every 2 years if normal. When amplification is not adequate, surgical intervention by someone experienced with operating on OI patients can provide long-term gain. Large surgical series from Finland, Holland, and Sweden reported gains over 20 dB and improved bone conduction thresholds in 80% of operated ears, but highlighted the higher success rate in avoiding footplate fusion at experienced academic centers.[189] Cochlear implantation is an option for profound sensorineural hearing loss; when care is taken about the hypervascularity of OI middle ear mucosa, satisfactory results have been reported.[190]

DI is one of the most consistent features of OI, occurring even in mosaic carriers with no other signs of OI.[41,171] The condition is almost always more severe in deciduous than in permanent teeth, varying in color from grayish translucent to yellow-brown.[191] Radiographically, there is a narrow or obliterated pulp chamber; histologically, the dentin layer is disorganized with a decreased number of scattered dentin tubules. Depending on the extent of wear and enamel fracture, the available options include composites combined with dental bonding agents for occlusal areas, fluoride-releasing glass ionomers for nonstressed areas and placement of crowns to maintain tooth vertical dimensions. Individuals with OI also have significant malocclusion, which is amenable to orthodontic intervention once permanent teeth have erupted.

For the past decade, antiresorptive bisphosphonates, especially pamidronate, have been administered to most children with OI and many adults. Bisphosphonates are widely used to treat osteoporosis in postmenopausal women. They are pyrophosphate analogs, which act via the inhibition of osteoclasts, and thereby decrease bone resorption. The rationale of this treatment in OI is that increasing trabecular bone volume and cortical thickness will increase resistance to long bone fracture, although the matrix will continue to contain mutant type I collagen.[192] These claims were not validated by the four controlled trials of bisphosphonate conducted in children with OI.[193–196] The decrease in long bone fractures in the controlled trials was equivocal in multiple meta-analyses,[197–199] and may represent a balance between increased cortical thickness and a decline in material properties of the treated bone.[148] The controlled trials were in agreement that bisphosphonate treatment improved vertebral geometry, showing new bone was resistant to compressive forces. However, despite improved vertebral compressions, retrospective analysis showed no improvement in the prevalence of scoliosis at maturity in children with collagen structural mutations, whether they received bisphosphonate before or after age 5 years, or not at all.[200] Studies focused on bone histology and BMD agreed that the maximum gain was achieved in 2–4 years of treatment.[194,201] Uncontrolled trials have also made claims for increased muscle strength and decreased bone pain, which have not been supported in the controlled trials. Concerns have also arisen about the cumulative dose effect of bisphosphonates, which have a decade-long half-life in bone, including impaired healing of fractures and osteotomies, adynamic bone with impaired remodeling due to toxic effects on bone cell populations and leading to increased microcracks and decreased mineralization heterogeneity, as well as delayed tooth eruption. For these reasons, there is great interest in short-acting antiresorptives, such as the RANKL-antibody denosumab which acts to reduce osteoclastogenesis, and anabolic drugs, such as antisclerostin antibody which stimulates osteoblasts to increase matrix production, which are currently being tested in animal models.[202,203]

Other therapies, which may have potential in the future, are modeled on the two naturally occurring clinical situations with mild phenotype, a null mutant allele, and parental mosaicism. One strategy for the conversion of

a mutant allele into a null allele involves targeted suppression of mutant collagen mRNA by hammerhead ribozymes or shRNA, which have been shown to down-regulate expression of the mutant collagen allele with relative selectivity and reduce the amount of mutant protein produced by OI patient cells in culture.[204,205]

An alternative therapeutic strategy involves the introduction of normal stem cells, which in principle have the potential to differentiate into normal osteoblasts and improve the ratio of normal to mutant collagen produced in OI patient bone. This approach would result in a molecular phenocopy of mosaics for collagen mutations, who have a relatively high-level of mutant osteoblasts but, nevertheless, have a mild OI phenotype.[69] In the Brtl mouse, adult bone-marrow cells were transplanted in utero and rescued Brtl perinatal lethality, as well as improving bone geometry and strength.[206] This improvement occurred, despite the fact that less than 2% of the cells in the recipient bone were of donor origin. A similar study in the *oim* mouse transplanted normal bone marrow stromal cells into developing *oim* femurs, and produced an improvement in bone parameters, again despite low levels of donor cell engraftment.[207] Combining this approach with the mild phenotype of a null collagen allele, patient mesenchymal cells have been targeted in vitro to inactivate the mutant type I collagen allele, and then reprogrammed to become pluripotent cells with the potential for osteogenesis once transplanted back into the patient.[208]

8 CONCLUSIONS

Although considerable progress has been made in recent years with the elucidation of genes and pathways involved in recessive OI, the complete picture is still far from clear. Phenotypic variations between individuals with identical mutations suggest the involvement of complex modifying effects, either genetic, epigenetic, or environmental, such as polymorphic changes in modifier genes which may change an individual's capacity to cope with the disease causing mutation(s) at a biochemical or cellular level. Clinical similarities between dominant and recessive OI types suggest that the ultimate molecular pathology may lie in a defective ECM, resulting in abnormal mineralization.

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CHAPTER

24

Osteoarthritis: Genetic Studies of Monogenic and Complex Forms

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1 BRIEF CLINICAL DESCRIPTION

Osteoarthritis (OA), the most common joint disorder worldwide,[1] is a chronic arthropathy in which cartilage loss, osteophyte formation, and subchondral bone sclerosis lead to pain, disability, and a reduction in quality of life.[2] Structural changes visible on radiography include narrowing of the joint space, osteophytes (bony projections at the periphery of the joint surface), and bone remodeling around the joints. OA can arise in any synovial joint, but is most common in the large joints (knees and hips), hands, and spine.[2] OA of the large weight-bearing joints (hips and knees) is the major contributor to 57,000 knee and 55,000 hip arthroplasties undertaken each year in the United Kingdom[3] and over 500,000 hip and knee arthroplasties per year in the United States.[4]

1.1 Diagnosis

OA is classified as an idiopathic or secondary to anatomic abnormalities, trauma, or inflammatory arthritis.[5] The American College of Rheumatology (ACR) criteria developed for hand, hip, and knee OA are intended to distinguish OA from other causes of joint symptoms and are best suited to clinical settings in which a high prevalence of other arthritides and joint pain is expected. This is different from the definition of OA used for epidemiological studies given the poor correlation between radiographic disease severity (joint damage) and the joint pain and functional impairment presented by a patient. Thus, OA can be defined pathologically, radiographically, or clinically, but most epidemiological studies have relied upon radiographic features to define the disease.[5]

The most widely used system to grade radiographic OA is the Kellgren and Lawrence (K/L) system,[6] which assigns a value from 0 to 4 based upon atlas reproductions (0 = normal; 1 = possible osteophytes; 2 = definite osteophytes and possible joint space narrowing; 3 = definite or multiple osteophytes, definite joint space narrowing, and some sclerosis; and 4 = large osteophytes, marked joint space narrowing, and severe sclerosis). Importantly, this system is "osteophytes driven" and hence unreliable when there is definite cartilage loss in the absence of osteophytes, as is often the case for hip OA.[5] In this case, a measure of minimum joint space width can be used but this may also differ between ethnic groups and age groups.[7] Although, MRI is increasingly common in epidemiologic studies, a universally agreed MRI definition for OA has not yet been established.

1.2 Mortality of OA

A recent large population-based study has found that individuals with OA (defined both symptomatically and radiographically) at the knee or the hip show a 55% excess in all-cause mortality.[6] Diabetes (95% increased risk), cancer (128% increased risk), cardiovascular disease (38% increased risk), and the presence of walking disability at baseline (48% increased risk) are independently associated with the excess of all-cause mortality.[6] Importantly, deaths from cardiovascular causes are higher in patients with walking disability due to OA (72% higher) even after adjustment for baseline covariates, indicating an interplay of OA with the additional comorbid conditions that results in a higher risk of mortality. Thus, although the main symptoms of OA are pain and disability, the consequences are far reaching.

1.3 Economic burden of OA

OA has a very high economic burden attributable to the effects of disability, comorbid disease, and expense of

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treatment.[7,8] Although, typically associated with less severe effects on quality of life and per capita expenditures than rheumatoid arthritis, OA is nevertheless a more costly disease because of its far higher prevalence, estimated to be approximately 12% of the adult population.[7] A recent US-based study has found that OA contributes substantially to health care expenditures. Among women, OA increased out-of-pocket expenditures by USD 1379 per year (2007 dollars) and insurer expenditures by USD 4833. Among men, OA increased out-of-pocket expenditures by USD 694 per annum and insurer expenditures by USD 4036. OA was estimated to raise aggregate annual medical care expenditures in the United States by USD 185,500,000,000.[8] Furthermore, the burden of OA is increasing. Even though direct and indirect per capita costs for OA have stabilized in recent years, the escalating prevalence of the disease, partly due to the increase in obesity and the median age of the population, has led to much higher overall spending for OA.[7]

1.4 Risk factors

Several risk factors affect hip and knee OA, namely body weight, age, female gender, occupational activity and injury,[5,9] congenital abnormalities and joint shape, meniscal tears, presence of OA at other joints (Heberden's and Bouchard's nodes), and foot and knee alignment in addition to genetic predisposition.[5,9,10] Furthermore, specific inter- and intraarticular patterns of OA may represent subsets that have different risk factor profiles and disease course.[5,10] The reader is referred to[5] for an in depth review of the epidemiology of OA.

At the level of articular cartilage, the pathological progression of OA follows a typical pattern.[11,12] The earliest indication of pathological change is chondrocyte clustering as a result of the increased proliferation of these cells and a general upregulation of their synthetic activities.[11] Increased expression of cartilage-degrading proteinases and matrix proteins by chondrocytes suggests an attempt at repair. Gradual loss of proteoglycans appears in the surface region of articular cartilage and this is followed by type II collagen degradation.[12] Cracks develop along the articular surface in the cartilage, producing the histological image termed as fibrillation. At later stages of the disease, fibrocartilage forms, probably, as a consequence of unsuccessful attempts by chondrocytes to fill in the cracks. Finally, the development of osteophytes is observed.[11] In addition to articular cartilage, synovium, tendons, and bone are also involved in OA progression and manifestation.[12] As discussed later, some of the genetic associations reported to date relate to these tissues (Fig. 24.1).

As of today, there is no disease modifying drugs for OA and treatment options either address symptoms only or involve joint surgery (Section 6). Thus, understanding the molecular mechanisms responsible for OA in order to develop diseases-specific interventions is of utmost importance. This unmet medical need gives added impetus to the genetic studies that can help elucidate the molecular pathogenesis of OA.

2 GENETICS DESCRIPTION

Understanding the genetic contribution to OA has two important clinical implications. First, the identification of genes involved in disease risk can improve our understanding of the molecular mechanisms involved in the pathogenesis of OA. Second, by selecting sets of genetic variants associated with risk of disease or with progression of OA, it should be possible to better define OA subphenotypes and to improve the risk assessment of OA in these various groups. As illustrated in Fig. 24.2, genetic variation can contribute to risk of OA and its subphenotypes at various stages of the disease. Genetic factors have been implicated in several of the risk factors that contribute to OA, such as inflammation, obesity, bone mineral density, skeletal shape, and are known to influence risk of hip, knee, and generalized OA.[13] Furthermore, OA progression measured radiographically or as cartilage loss by MRI is under genetic control[14,15] and sensitivity to pain, which is the major clinical outcome of OA is also strongly influenced by genetic factors.[16]

2.1 Familial Aggregation and Heritability of OA

OA runs in families. This is measured using the risk ratio for a relative, such as a brother or sister, of an affected individual to that of the general population prevalence.[16] For affected sib pairs, this sib recurrence risk is called the lambda sib (λs). In fact, when the occurrence of OA is tested in siblings of individuals with OA severe enough to lead to total joint replacement (TJR) in comparison to the general population, it is found that these sibs have a significantly higher prevalence of OA. The λs for knee OA/total knee replacement has been estimated to be 2.08–4.80 and for hip OA/total hip replacement is 1.78–8.53.[17]

An alternative method to assess the genetic contribution to a disease, in this case OA, is twin studies which enable investigators to quantify the environmental and genetic factors that contribute to a trait or disease.[16] Comparing the concordance for disease status in identical twins with the concordance for disease status in nonidentical twins quantifies the proportion contributed by genetic variation to a given trait or disease. This is called "heritability." The heritability of OA has been calculated in twin sets after adjustment for other known risk factors, such as age, sex, and body mass index. The findings show that the influence of genetic factors in radiographic OA of the hand, hip, and knee in women is between 39% and 65%.[16] Twin studies and familial aggregation studies

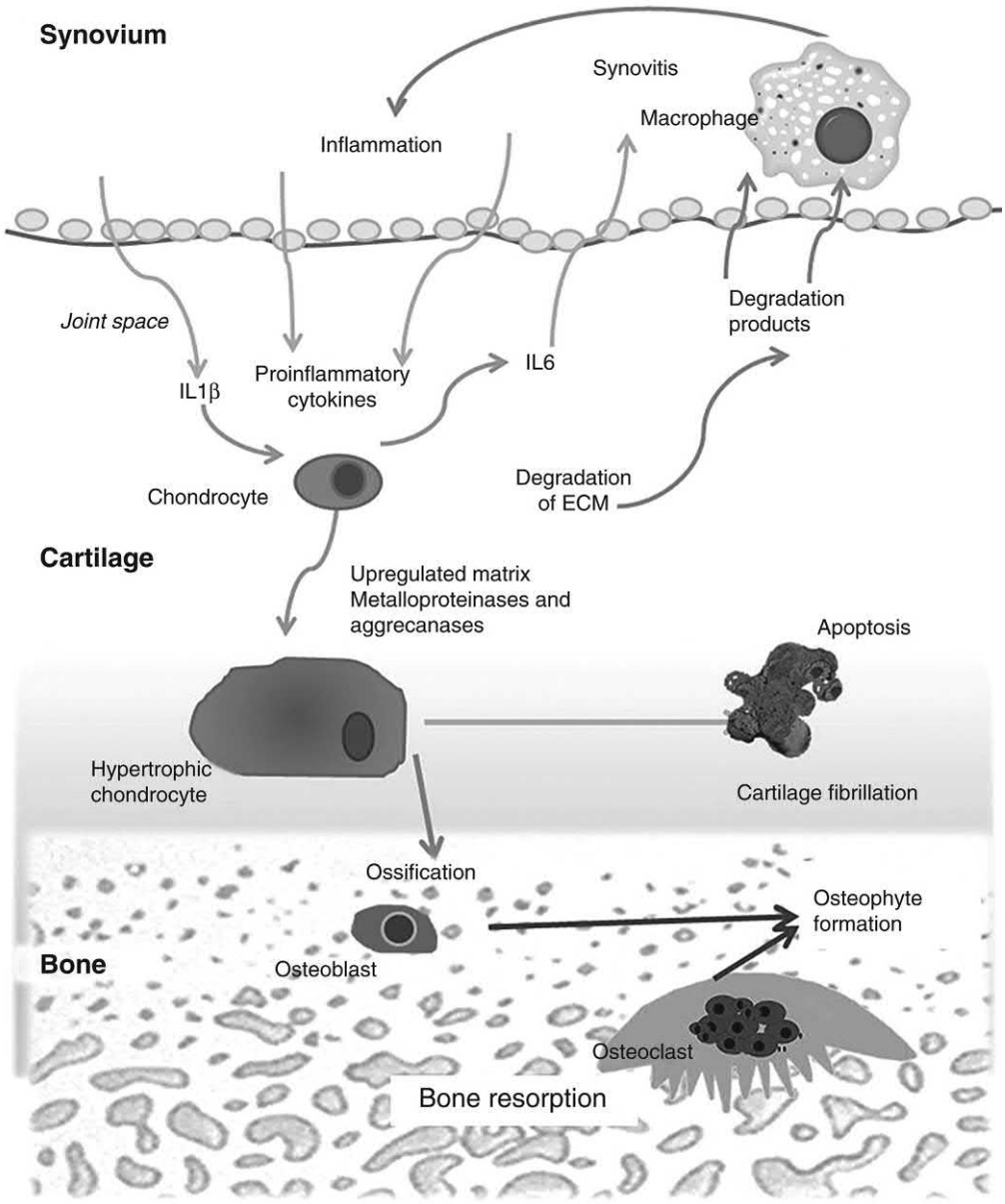

FIGURE 24.1 Schematic representation of some of the biological changes that take place in synovium, articular cartilage and subchondral bone during the course of osteoarthritis (OA).

have also investigated the genetic contribution to cartilage volume and progression of OA.[14,15]

3 MOLECULAR GENETICS

The reference numbers in the online mendelian inheritance in man (OMIM) database for the disorders discussed in this section are presented in Table 24.1.

3.1 Monogenic Syndromes

Some characteristics of OA, such as articular chondrocyte proliferation, their expression of hypertrophy markers [e.g., matrix metalloproteinase 13 (MMP-13) and collagen X], remodeling of the cartilage matrix by proteases, vascularization, and focal calcification of joint cartilage with calcium hydroxyapatite crystals—resemble chondrocyte differentiation processes during skeletal development mediated by endochondral ossification.[18] Based on this observation, it has been proposed that signaling molecules regulating chondrocyte activities in growth cartilage may also be involved in the pathogenesis of OA.[19] Endochondral ossification is initiated by the formation of cartilage templates of future bones, built by mesenchymal progenitor cells, which differentiate into chondrocytes, and then the differentiated cartilage cells undergo a cascade of late differentiation events

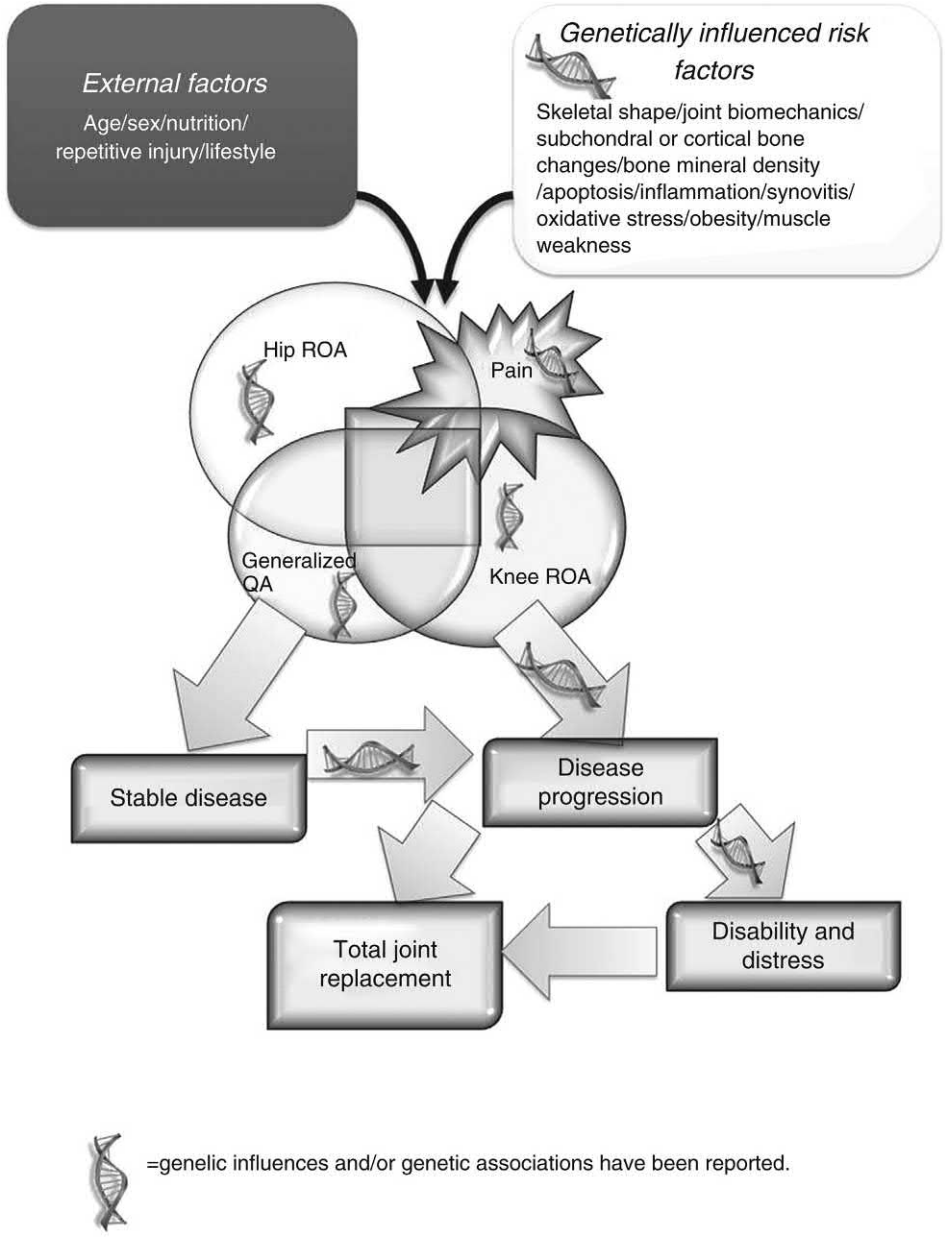

FIGURE 24.2 Genetic variation influences osteoarthritis risk and related clinical phenotypes at various stages during the course of OA. Genetic variation can influence factors that in turn affect risk of OA or can affect directly the risk of progression and clinical presentation of the disease.

culminating in chondrocyte hypertrophy. After invasion of blood vessels from the subchondral bone, the majority of hypertrophic cells undergo apoptosis and the cartilage template is remodeled into trabecular bone.[18] Similarly, proliferation of chondrocytes, hypertrophic differentiation of chondrocytes, remodeling and mineralization of the extracellular matrix, invasion of blood vessels, and apoptotic death of chondrocytes correspondingly occur during OA.[11,18]

The similarities in the pathologic progression of the disease, even when the initiating events are different, suggest that there may be a common molecular sequence of events underlying OA progression.[18] Accordingly, to understand the molecular basis of OA, there has been a major emphasis to understand rare monogenic disorders with features that resemble OA.[11]

Premature OA is in fact a part of the pathology associated with many mutations affecting cartilage extracellular assembly or stability, such as Stickler's syndrome type I, multiple epiphyseal dysplasia, and osteochondritis dissecans[11,18] (Table 24.1).

Many other disorders that influence skeletal development are not discussed here, as their presentation is neither OA-like nor do they result in early onset OA.

TABLE 24.1 Select Monogenic Disorders That Display Early Onset OA or OA-Like Symptoms

Names of disorders	Mode of inheritance	MIM no.	Chromosomal regions	Genes
ACG2; Langer–Saldino	Autosomal dominant	200610	12q13.1	*COL2A1*
Platyspondylic dysplasia, torrance type	Autosomal dominant	151210	12q13.1	*COL2A1*
Hypochondrogenesis	Autosomal dominant	200610	12q13.1	*COL2A1*
SEDC	Autosomal dominant	183900	12q13.1	*COL2A1*
SEMD Strudwick type	Autosomal dominant	184250	12q13.1	*COL2A1*
Kniest dysplasia	Autosomal dominant	156550	12q13.1	*COL2A1*
Spondyloperipheral dysplasia	Autosomal dominant	271700	12q13.1	*COL2A1*
Mild SED with premature onset arthrosis	Autosomal dominant		12q13.1	*COL2A1*
Stickler syndrome type 1	Autosomal dominant	108300	12q13.1	*COL2A1*
Stickler-like syndrome				
Stickler syndrome type 2	Autosomal dominant	604841	1p21	*COL11A1*
Marshall syndrome	Autosomal dominant	154780	1p21	*COL11A1*
OSMED, recessive type	Autosomal recessive	215150	6p21.3	*COL11A2*
OSMED, dominant type (Weissenbacher–Zweymüller syndrome, Stickler syndrome type 3)	Autosomal dominant	215150	6p21.3	*COL11A2*
PSACH	Autosomal dominant	177170	19p12-13.1	*COMP*
MED type 1 (EDM1)	Autosomal dominant	132400	19p13.1	*COMP*
MED type 2 (EDM2)	Autosomal dominant	600204	1p32.2-33	*COL9A2*
MED type 3 (EDM3)	Autosomal dominant	600969	20q13.3	*COL9A3*
MED type 5 (EDM5)	Autosomal dominant	607078	2p23-24	*MATN3*
MED type 6 (EDM6)	Autosomal dominant	120210	6q13	*COL9A1*
DMC	Autosomal recessive	223800	18q12-21.1	*DYM*
PPRD	Autosomal recessive	208230	6q22-23	*WISP3*
SED Kimberley type	Autosomal dominant	608361	15q26.1	*AGC1*
SEMD, Aggrecan type	Autosomal recessive	612813	15q26	*AGC1*
SEMD, PAPSS2 type	Autosomal recessive	603005	10q23-q24	*PAPSS2*
SEMD Matrilin type	Autosomal recessive	608728	2p23-p24	*MATN3*
SEMD Missouri type	Autosomal dominant	602111	11q22.3	*MMP13*
SED tarda, X-linked (SED–XL)	X-linked recessive	313400	Xp22	*SEDL*
Familial osteochondritis dissecans	Autosomal dominant	165800	15q26	*AGC1*

ACG2, Achondrogenesis type 2; DMC, Dyggve–Melchior–Clausen dysplasia; MED, multiple epiphyseal dysplasia; MIM, Mendelian inheritance in man number; OSMED, otospondylomegaepiphyseal dysplasia; PPRD, progressive pseudorheumatoid dysplasia; PSACH, pseudoachondroplasia; SED, spondyloepiphyseal dysplasia; SEDC, spondyloepiphyseal dysplasia congenital; SEMD, spondyloepimetaphyseal dysplasia.

The following is only a brief summary, and for more detailed information the reader is referred to other chapters in this book dealing with monogenic disorders of skeletal development and to the review by Warman and coworkers in 2011.[19] A partial list of monogenic disorders that manifest OA or OA-like findings is shown in Table 24.1. Brief descriptions of some of these diseases are as follows.

3.1.1 Osteochondritis Dissecans

Osteochondritis dissecans is a common cause of joint pain and dysfunction in children and young adults.[20] Defined as a separation of cartilage and subchondral bone from the surrounding tissue; it primarily affects the knee, ankle, and elbow joints. Repetitive microtrauma is thought to be its pathogenesis.[20] However, other causes, including acute trauma, ischemia, ossification

abnormalities, and genetic factors, have also been proposed.[21] Most cases of osteochondritis dissecans are sporadic, with a prevalence of knee osteochondritis of 15–29 per 100,000.[21] Familial osteochondritis dissecans is a rare disorder (OMIM #165800) involving disturbed chondroskeletal development, disproportionate growth, and deformation of the skeleton.[22] It is presented as multiple osteochondritic lesions in knees and/or hips and/or elbows, disproportionate short stature, and early OA. A genome-wide multipoint linkage analysis of a Swedish family displaying the disease over five generations, (segregating in an autosomal-dominant fashion) led to the identification of a heterozygous mutation in the aggrecan C-type lectin domain (*ACAN*) gene causing this disorder indicating a defect in the extracellular matrix.[22]

3.1.2 Multiple Epiphyseal Dysplasia

Multiple epiphyseal dysplasia (MED) (OMIM #226900, #132400, #607078, #600969, and #600204) is a relatively mild and clinically variable osteochondrodysplasia, primarily characterized by delayed and irregular ossification of the epiphyses, and early-onset OA.[11] Mutations in the genes encoding cartilage oligomeric matrix protein (*COMP*) and type IX collagen (*COL9A2* and *COL9A3*) cause different forms of MED[23,24] (Table 24.1). An autosomal recessive form of MED (EDM4) is caused by a mutation in the diastrophic dysplasia sulfate transporter gene (*SLC26A* also called *DTDST*).[25] A genome-wide screen of family with autosomal dominant MED not linked to the EDM1-3 genes provides evidence for a MED locus on the short arm of chromosome 2 (2p24-p23), and a search for a candidate gene identified *MATN3*,[26] which was later supported by animal model mutations.

3.1.3 Pseudoachondroplasia

Pseudoachondroplasia (PSACH) (OMIM #177170) is a disproportionate dwarfing condition associated with joint abnormalities that maps to chromosome 19p12-13.1.[23] Most cases appear to have an inherited autosomal dominant trait. Affected individuals may have short, stubby fingers (brachydactyly), and bowed legs (genu varum), or "knock knees" (genu valgum). Additionally, spinal abnormalities include increased curvature of the lower vertebrae (lumbar lordosis) and anterior flexion of the thoracis spine (kyphosis). Cartilage oligomeric matrix protein (COMP), a cartilage specific protein, maps to the same location within a contig that spans the PSACH locus. Subsequently, Hecht et al.[23] identified COMP mutations in multiple unrelated patients, showing that mutations in the COMP gene cause PSACH.

3.1.4 Spondyloepiphyseal Dysplasia Tarda

Spondyloepiphyseal dysplasia tarda (SEDT; SEDL) (OMIM #313400) is an X-linked late-onset progressive skeletal disorder that impairs bone growth and manifests almost exclusively in males. It manifests in childhood and is characterized by disproportionate short stature with a short trunk, barrel chest, but absence of systemic complications.[27] Mutations in the trafficking protein particle complex C2 protein (TRAPPC2), a mammalian ortholog of yeast Trs20p and a component of the trafficking protein particle (TRAPP) vesicle tethering complex, have been reported to cause SEDT.[27] The *TRAPPC2* gene product (also called *SEDLIN*) is present in the nucleus and forms homodimers. SEDT-associated mutations interfere with their interaction with transcription factors.[28]

3.1.5 Otospondylomegaepiphyseal Dysplasia

Otospondylomegaepiphyseal dysplasia (OSMED) (OMIM #215150) is an autosomal recessive trait characterized by dysplasia, hearing loss, distinct facial features, and often times early-onset polyarticular OA.[29] Skeletal malformations affect the bones of the arms, legs, and spine resulting in disproportionate short stature. Hearing loss is often severe. Intelligence is normal. Homozygous OSMED result from mutations in the *COL11A2* gene.[29] Two additional allelic disorders, Weissenbacher–Zweymuller syndrome[30] and Stickler syndrome III,[31] are also caused by mutations within this gene (Table 24.1).

3.1.6 Stickler Syndrome

Stickler Syndrome (OMIM #108300, #604841, #609508, #184840) also called hereditary arthroophthalmopathy, is an autosomal dominant multisystem disorder that can affect the eyes, ears, skeleton and joints, and craniofacies. Complications may include myopia, cataract, and retinal detachment; hearing loss that is both conductive and sensorineural; midfacial underdevelopment and cleft palate; and mild spondyloepiphyseal dysplasia and/or arthritis. Stickler syndrome is associated with mutations in three genes: *COL2A1* (chromosomal locus 12q13), *COL11A1* (chromosomal locus 1p21), and *COL11A2* (chromosomal locus 6p21).[32–34] Most cases are due to *COL2A1* mutations. All *COL2A1* mutations known to cause Stickler syndrome result a premature termination codon. Mutations in *COL11A1* have only recently been described, and *COL11A2* mutations have been identified only in patients lacking ocular findings. Although Stickler's syndrome is associated with mutations in the *COL2A1, COL11A1*, and *COL11A2* genes, yet no linkage to any of these three loci can be established in some rare patients with clinical findings consistent with Stickler syndrome. It is presumed that mutation of additional genes can also account for Stickler syndrome.[32–34]

3.1.7 Marshall Syndrome

Marshall syndrome (OMIM #154780), usually, recognized from its facial features, which include an upturned nose, eyes spaced widely apart (making them appear larger than normal), and a flat nasal bridge. Patients

may also have abnormalities of the palate. In addition, patients may experience early OA, particularly in the knees. Myopia, cataracts, and glaucoma are also common. Moderate-to-severe hearing loss is often preceded by recurrent otitis media, sometimes in children as young as 3 years of age. Marshall syndrome may be a subset of Stickler syndrome,[34] because individuals with both syndromes have similar facial features and symptoms. Both Stickler and Marshall syndromes are autosomal dominant chondrodysplasias characterized by midfacial hypoplasia, high myopia, and sensorineural-hearing deficit. Some patients, however, present with phenotypes of both Marshall and Stickler syndromes[34] and the characteristics of these disorders overlap. Hence, it has been argued that they represent a single entity. On the other hand, most patients with Stickler syndrome have cataracts, while this problem is less common among those with Marshall syndrome.[33] In addition, most individuals with Marshall syndrome have moderate to severe hearing loss, which rarely occurs in Stickler syndrome individuals, who have normal hearing. Patients with Marshall syndrome were much more likely to have short stature than those with Stickler syndrome.[34,35] Mutations within *COL11A1* cause Marshall syndrome.[36] Several mutations causing Stickler syndrome have been found in the *COL2A1* gene, and one mutation causing Stickler syndrome and one causing Marshall syndrome have been detected in the *COL11A1* gene. Mutation analysis of patients with Stickler, Stickler-like, or Marshall syndrome identified dozens of different mutations involving the three genes, *COL11A1*, *COL11A2*, and *COL2A1*. Genotypic-phenotypic comparison revealed an association between the Marshall syndrome phenotype and splicing mutations of the *COL11A1* gene. Null-allele mutations in the *COL2A1* gene lead to a typical phenotype of Stickler syndrome.

3.1.8 Progressive Pseudorheumatoid Displasia

Progressive pseudorheumatoid dysplasia (OMIM #208230) is an inherited skeletal dysplasia with radiographic changes notably in the spine, similar to spondyloepiphyseal dysplasia tarda.[37] There is also an articular cartilage involvement, which engenders some clinical resemblance to rheumatoid arthritis.[37] The disease is characterized by continuous degeneration and loss of articular cartilage due to mutations in the gene that encodes *WISP3*. These mutations cause a series of cellular and molecular changes that disturb endochondral ossification.[38]

3.1.9 Dyggve–Melchior–Clausen Syndrome and Smith–McCort Dysplasia

Dyggve–Melchior–Clausen syndrome and Smith–McCort dysplasia (OMIM #223800, #607326) are recessive spondyloepimetaphyseal dysplasias caused by loss-of-function mutations in the dymeclin (*DYM*), a gene with previously unknown function. Osipovich et al.[39] described the genetic and proteomic analysis of mammalian dymeclin, a gene until then of unknown function. The mutant allele in the dymeclin gene that they generated, structurally resembled the mutation observed in human cases of Dyggve–Melchior–Clausen syndrome, and encoded a protein in which the carboxy-terminal 49 amino acids have been replaced by residues of an irrelevant sequence. Dym-deficient mice developed osteochondrodysplasia similar to the human forms. The Dym protein was expressed at highest levels in the Golgi, associated with cellular proteins involved in vesicular traffic to and from the Golgi complex. Dym-mutant fibroblasts displayed multiple defects in vesicular traffic. Dymeclin was shown to participate in the traffic of vesicles and proteins into and out of the Golgi and to be related to osteochondrodysplasias in mice and men, thus establishing the importance of intracellular vesicle traffic in mammalian postnatal bone formation, a highly organized process involving chondrocyte proliferation, differentiation, apoptosis, and calcification.[40]

3.1.10 Spondyloepiphyseal Dysplasia

Spondyloepiphyseal dysplasia (SED) (OMIM #143095) encompasses a heterogeneous group of disorders characterized by shortening of the trunk and limbs.[41] The autosomal dominant SED type Kimberley (SEDK) causes premature degenerative arthropathy and was mapped in a multigenerational family to the *AGC1* gene, which encodes aggrecan.[41] On the other hand, the study of two large consanguineous families from Oman with a distinct form of SED (SED Omani type) revealed a missense mutation in the gene that encodes chondroitin 6-O-sulfotransferase (C6ST-1) gene (*CHST3*).[42]

3.1.11 Spondyloepimetaphyseal Dysplasias

The *spondyloepimetaphyseal dysplasias* (SEMDs), a heterogenous group of skeletal diseases featuring defective growth and modeling of the spine and long bones,[19] often arise sporadically, but distinctive forms with autosomal dominant, autosomal recessive, or X-linked transmission have been reported.[43,44] Radiological and histopathological studies have indicated that the SEMDs likely reflect fundamental disturbances in growth plate development.[45] Pedigree analyses have identified genetic defects in at least four autosomal SEMDs: the dominant Strudwick type, the recessive Pakistani or PAPSS2 type, recessive matrilin 3 deficiency, and dominant Missouri type.[24,46–48] Strudwick type SEMD is caused by missense mutations of the *COL2A1* gene.[44] PAPSS2 type SEMD is due to a mutation of the 3′-phosphoadenosine 5′-phosphosulfate synthase 2 gene (*PAPSS2* or *ATPSK2*), located on chromosome 10q23-34. This mutation likely impairs posttranslational sulfation of cartilage

extracellular matrix (ECM), causing defects in maturation and growth plate function.[47] The matrilin 3 gene is located on chromosome 2p24–25, and homozygosity for a missense mutation caused SEMD in a consanguineous family.[24] Genome-wide linkage analysis mapped the autosomal dominant Missouri type SEMD to a 17-cM region on chromosome 11q14.3-23.2 that contains a cluster of 9 MMP genes. Among these, MMP13 represented the best candidate, as it preferentially degrades collagen type II. By modeling MMP13 structure, Kennedy et al.[48] found that the MMP13 F56S mutation would result in a hydrophobic cavity with misfolding, autoactivation, and degradation of mutant protein intracellularly. Expression of wild type and mutant MMP13s in human embryonic kidney cells confirmed abnormal intracellular autoactivation and autodegradation of F56S MMP13 such that only enzymatically inactive, small fragments were secreted. Thus, the F56S mutation results in deficiency of MMP13, which leads to the human skeletal developmental anomaly of autosomal dominant Missouri type of SEMD.[48]

Although, the list of disorders in Table 24.1 is not comprehensive, the genes represented by these monogenic OA-related diseases merit some discussion. Most of these genes are either structural components of cartilage (collagen type 2, collagen type 11, matrilin 3, cartilage oligomeric matrix protein) or are implicated in cartilage metabolism (MMP13). In fact, different mutations in the same gene can give rise to different disorders. For example, *COL2A1* mutations cause at least eight type 2 collagenopathies, including spondyloepiphyseal dysplasia (SED) congenita, SED Namaqualand type, mild SED with premature onset arthrosis, Stickler dysplasia type I, spondyloperipheral dysplasia, Kniest dysplasia, hypochondrogenesis, and achondrogenesis II; and matrilin 3 mutations cause forms of MED[19] (Table 24.1).

Cartilage oligomeric matrix protein (COMP) is a noncollagenous ECM expressed primarily in cartilage, ligaments, and tendons and mutations in *COMP* cause pseudoachondroplasia and MED. Both of these skeletal dysplasias have a characteristic chondrocyte pathology that consists of intracellular retention of COMP and other ECM proteins in an enlarged rough endoplasmic reticulum. This toxic intracellular retention of ECM proteins leads to chondrocyte death thereby decreasing linear bone growth. Additionally, when COMP and the other coretained proteins are not exported to the ECM, the resulting matrix is abnormal and easily erodes with normal physical activity.[49]

Aggrecan is another important cartilage component, such that structural mutations result in an OA phenotype. Aggrecan contributes an extreme anionic charge density to the ECM, which by osmotic effects leads to water retention and swelling, resisted by the tensile properties of the collagen fibers. Other important interactions are those with cell surface receptors, such as integrins, heparan sulfate proteoglycans, hyaluronan receptors, and others.[50]

Mutations in the gene encoding the matrilin 3 protein (*MATN3*), cause monogenic syndromes. Matrilin 3 is one of the families of 4 oligomeric ECM proteins, and disruption of possible interactions with other proteins, such as collagen types 2 and 9, may destabilize the ECM structure.

MMP-13 has long been considered as the major enzyme involved in OA cartilage erosion.[12] Suggestive evidence was obtained by transgenic postnatal overexpression of the enzyme in mice, resulting in focal OA cartilage pathology at load bearing sites.[51] Experiments in surgically-induced OA in mice have shown that MMP-13 deficiency can inhibit cartilage erosion in the presence of aggrecan depletion, and that aggrecan depletion on its own does not drive cartilage erosion. This implies that aggrecan loss paves the way for MMP-13 mediated attack of denuded collagen type II.[52] MMP-13 knockout mice demonstrated that MMP-13 has no critical role in OA-associated osteophyte formation. Osteophytes and chondrocyte hypertrophy (as characterized by excess levels of collagen type 10) developed undisturbed in the absence of MMP-13.[52]

Most of these findings match current knowledge of human cartilage biology and of mouse models of OA.[11] By contrast, none of the genes encoding such molecules has been shown to consistently contribute to genetic risk for the common form of OA (Table 24.2).

A molecular mechanism to come out of monogenic studies is that of traffic of vesicles and proteins into and out of the Golgi complex via the study of dymeclin (Dym) in recessive spondyloepimetaphyseal dysplasia.[39] The Dym protein is expressed at highest levels in the Golgi, associated with cellular proteins involved in vesicular traffic to and from the Golgi complex.[40] This demonstrating the relevance of transport defects in endochondral bone formation. This is of interest as one of the genes to come out of genome-wide association studies (GWAS) is the component of oligomeric golgi complex 5 (*COG5*) gene.[58] The encoded protein is required for normal Golgi morphology and function, suggesting that this could be a shared mechanism between monogenic and complex forms of OA.[58]

3.2 Genetic Association Studies and Genome-Wide Scans

3.2.1 Candidate Gene Studies

Genetic association analysis of candidate gene regions without any preceding linkage analysis has a long history of discovering single-marker disease allele associations.[13] A number of candidate gene studies for knee and hip OA were carried out in the past with varying

TABLE 24.2 Genetic Associations With OA Achieving At Least $P < 1 \times 10^{-7}$

SNP id	Genes	Ethnic groups	Traits	Total sample size[a]	*p*-Value	Putative or known functions	References
rs143383	*GDF5*	Asian	Hip OA	998 P; 983 C	2×10^{-13}	Bone morphogenetic protein and joint development	53
rs11718863	*DVWA*	Asian	Knee OA	982 P; 1774 C	7×10^{-11}	Cartilage-specific tubulin binding	54
rs11177/ rs6976[b]	*GLN3/GLT8D1*	Caucasian	Hip or knee OA	14,883 P; 53,947 C	7×10^{-11}	Cell cycle control, tumorigenesis, and cellular senescence	55
rs4836732	*ASTN2*	Caucasian	THR	5,813 P; 53,947 C	6.1×10^{-10}	Glial neuronal migration	55
rs9350591	*FILIP1/SENP6*	Caucasian	THR	5,813 P; 53,947 C	2×10^{-9}	Genetic signal mapping to a region covering more than one gene	55
rs10947262	*BTNL2*	Asian	Knee OA	906 P; 3396 C	5×10^{-9}	Immunomodulatory function and T-cell response	56
rs143383	*GDF5*	Caucasian	Knee OA	6,861 P; 10,103 C	8×10^{-9}	Bone morphogenetic protein and joint development	57
rs4730250	*COG5/GPR22/ DUS4L/ HBP1*	Caucasian	Knee OA	6,709 P; 44,439 C	9×10^{-9}	Genetic signal mapping to a region covering more than one gene	58
rs11842874	*MCF2L*	Caucasian	Knee or hip OA	19,041 P; 24,504 C	2×10^{-8}	Cell motility	59
rs10492367	*PTHLH*	Caucasian	Hip OA	6,329 P; 53,947 C	1.5×10^{-8}	Chondrogenic regulator	55
rs835487	*CHST11*	Caucasian	THR	5,813 P; 53,947 C	2×10^{-8}	Chondroitin sulfotransferase involved in cartilage metabolism	55
rs7775228	*HLA–DQB1*	Asian	Knee OA	906 P; 3396 C	2×10^{-8}	Immune response (antigen presentation)	56
rs12107036	*TP63*	Caucasian	TKR in women	4,085 P; 33,587 C	7×10^{-8}	Member of the p53 family of transcription factors	55
rs8044769	*FTO*	Caucasians	TKR in women	4,085 P; 33,587 C	7×10^{-8}	Control of energy homeostasis	55
rs10948172	*SUPT3H/ RUNX2*	Caucasians	OA (hip or knee) in men	5,617 P; 20,360 C	8×10^{-8}	Probable transcriptional activator	55
rs6094710	*NCOA3*	Caucasians	Hip OA	11,277 P; 67,473 C	7.9×10^{-9}	Nuclear receptor	60
rs788748	*IGFBP3*	Caucasians	Hip OA	3243 P; 6891 C	2×10^{-8}	Cartilage catabolism and osteogenic differentiation	61
rs12982744	*DOT1L*	Caucasians	Hip OA	9,789 P; 31,873 C	8.1×10^{-8}	Wnt signaling	62
rs3204689	*ALDH1A2*	Caucasians	Hand OA	2,648 P; 79,690 C	5.6×10^{-12}	Metabolism of retinoic acid	63

THR, Total hip replacement; TKR, total knee replacement.
[a] *P = number of patients; C = number of controls.*
[b] *These SNPs are in total linkage disequilibrium with each other.*

levels of success. This approach is limited to that, and it relies upon a priori understanding of the etiology of OA and allows for only very small regions of the genome to be targeted for investigation. A comprehensive list of all the genetic loci reported to be associated with OA using the candidate method is not possible within this review. This approach has identified several important genes, such as *ASPN*, *GDF5*, and *SMAD3*, that continue to be compelling targets for functional studies and further genetic replication in independent populations.[13,17] A systematic approach to assess the role of OA candidate genes was used by Rodriguez-Fontanela and coworkers. The authors identified candidate genes using the Human Genome Epidemiology Navigator, and used genetic association data from existing genome-wide association scan (GWAS) of OA. They tested all the SNPs in 199 candidate genes for OA with a minor allele frequency over 5% in 9 GWASes, which amounted

to 5,636 knee OA versus 16,972 controls and 4,349 hip OA versus 17,836 controls, all of European ancestry. Results were replicated in an additional sample of 5921 individuals. Given the number of tests carried out the *p*-value for statistical significance was $P < 1 \times 10^{-5}$ after adjustment for multiple tests, and this left SNPs mapping to only two genes, *VEGF* and *COL11A1*, associated with OA.[64]

3.2.2 Genome-Wide Association Studies

With advances of high-throughput SNP genotyping technology, genome-wide association studies (GWAS) became possible in the past decade.[17] GWAS took advantage of linkage disequilibrium (LD); that is, the fact that in any given chromosomal region in the genome alleles at physically nearby loci segregate together in the population. The markers analyzed need not be functional, but may simply be in LD with the functional variant.[65] However, the large-scale nature of GWAS introduces a multiple testing issue requiring replication of any positive associations. GWAS are nevertheless a powerful approach for unlocking the genetic basis of complex diseases, such as OA. Individual studies may be hampered by sample size limitations, which result in lack of statistical power and metaanalyses based on consortium efforts may help to overcome some of these limitations.

Several GWAS in OA have been published to date and a summary of the findings is presented in Table 24.2. While they clearly show that there is no definitive and common highly penetrant allele that causes OA, some interesting genes have emerged. The findings better delineate the types of genes and genetic variants that are involved in OA and provide substantial insight for future research.

The first GWAS using over 100,000 markers to be published was, from the United Kingdom, using pooled DNAs from 357 knee OA patients and 285 controls.[66] It identified a signal (rs4140564) between *PTGS2* and *PLA-2G4A*.[66] A second GWAS of knee OA, was published by a Japanese group.[54] After screening patients and 658 controls for <80,000 SNPs, a subset of 2,153 SNPs were tested in a second set of 646 knee OA cases and 631 controls. Two variants, in this region were identified as the most strongly associated. The locus was named double von Willebrand factor A (*DWVA*), and two coding variants, rs11718863 and rs7639618, were confirmed to be strongly associated with the risk of knee OA, in addition to Chinese and Japanese cases and control samples. The two SNPs were found to influence the binding of the *DVWA* protein to β-tubulin, and the authors hypothesized that tubulins and microtubules might be protective factors in the pathogenesis of OA.[54] Subsequently, however, the two SNPs were found to show no association on knee or hip OA in Caucasian patients.[67] On the other hand, it was later shown bioinformatically that *DVWA* is a part of the human gene coding for the collagen VI alpha4 chain (*COL6A4*)[68] (Table 24.2).

Another Japanese study identified two SNPs within a small region of the HLA locus on chromosome 6p to be associated with knee OA, with $P < 7 \times 10^{-8}$.[56] However, replication was not achieved in European cohorts and a population of Han Chinese.[69,70]

GWAS in European cohorts from the Netherlands (the Rotterdam study)[71] identified a signal ($p < 8 \times 10^{-8}$, OR 1.14) in a region on chromosome 7q22 that included a large LD block extending over 500 kb associated with knee and hand OA. Addition of several more cohorts to the original study increased the credibility of this signal. However, the LD block contained six known genes, all of which are equally good candidates for association with OA. These include *PRKAR2B* (encoding protein kinase-cAMP-dependent-regulatory type II-β), *GPR22* (encoding G protein-coupled receptor 22), and *COG5* (encoding component of oligomeric golgi complex 5). A subsequent metaanalysis, which included 6,709 patients with knee OA and 44,439 controls, showed conclusively that this signal is associated with genome-wide significance in European-descent samples with OR = 1.17 (95% CI 1.11–1.24), and a *p*-value of 9.2×10^{-9}, but not in Asian populations, where the OR was 1.03; (95% CI 0.85–1.25; n.s.)[58] (Table 24.2).

The arcOGEN study is a UK consortium, based around seven collection centers, which reported a discovery cohort of 3177 patients and 4894 population (not phenotypically characterized) controls.[72] Replication of signals involved additional European cohorts, as well as Caucasian North Americans, resulting in an overall metaanalysis sample of 13,768 hip and knee OA patients and 53,286 controls. Although it involved the largest sample size to date, it was unable to identify a signal of genome-wide significance. The strongest signal was rs2277831 ($P = 2.3 \times 10^{-5}$), located within *MICAL3*.[72]

Later, the same group generated 1000 genomes project-based imputation[73] on the same data from the arcoGEN consortium as aforementioned (3177 knee and hip OA patients and 4894 population controls). Imputation methods exploit information on patterns of multimarker correlation (linkage disequilibrium) from publically available databases, such as the International HapMap project or the SeattleSNPs resequencing studies, and more recently the 1000 genomes project,[73] to estimate or "impute" individual patient or control genotypes at untyped SNPs, and assess the estimated genotypes for association with phenotype. The imputed data were then used to detect previously unidentified risk loci. Through large-scale replication, it was possible to establish robust association with SNPs in the MCF.2 cell line derived

transforming sequence-like (*MCF2L*).[59] The top signal rs11842874 reached a combined OR1.17 (95% CI 1.11–1.23), $P = 2.1 \times 10^{-8}$ across a total of 19,041 OA cases and 24,504 controls of European descent (Table 24.2). *MCF2L* encodes a rho-specific guanine nucleotide exchange factor, and its role in OA remains unclear.

A GWAS metaanalysis on 78,000 individuals identified a genome wide significant variant (rs6094710) in the *NCOA3* gene (OR = 1.28 95% CI 1.18–1.39; $P = 7.9 \times 10^{-9}$).[65] This *p*-value was improved after combined analysis of the discovery ($P = 5.6 \times 10^{-8}$) and follow-up studies ($P = 7.3 \times 10^{-4}$). Two loci remained suggestively associated: rs5009270 at 7q31 (OR = 1.10; $P = 9.9 \times 10^{-7}$) and rs3757837 (OR = 1.27; $P = 2.2 \times 10^{-6}$ in a male-specific analysis)[62] (Table 24.2).

A GWAS on hip OA, using data from the Osteoporotic Fractures in Men Study and the study of osteoporotic fractures, was replicated in five independent studies. The rs788748 SNP located near the *IGFBP3* gene was genome-wide significant in this analysis and associated with a lower risk of hip OA (OR = 0.71; $P = 2.0 \times 10^{-8}$). Although the association replicated in all five studies, the signal was weakened after replication, suggesting a possible false positive result (OR = 0.92; $P = 0.020$). Despite this, a role of this variant and gene in OA is suggested by the results of functional validation studies.[63] Further replication is necessary, ideally with larger sample sizes, to make a more confident assertion of this variant's role in OA[63] (Table 24.2).

A GWAS on cartilage thickness at the hip has been carried out using data from the Rotterdam study. A SNP in the DOT1L gene was strongly associated with mJSW at the hip[74]. After replication in independent UK cohorts, an overall genetic effect size (expressed as the regression coefficient beta) of 0.09 mm/allele was achieved ($P = 1.1 \times 10^{-11}$ after metaanalysis).[74] The risk allele for lower mJSW at this SNP was later associated with a 10% increased risk of hip OA ($P = 8.8 \times 10^{-8}$). This effect reached genome wide significance in males (OR = 1.17 95% CI 1.11–1.23; $P = 7.8 \times 10^{-9}$), but was only nominally significant in women with a small effect size (OR = 1.05), consistent with the sexual dimorphism seen in some forms of hip OA[66] (Table 24.2).

The first GWAS to report genome-wide significant results for hand OA revealed a SNP (rs3204689) in the *ALDH1A2* gene to be significantly associated with an increased risk of hand OA in an Icelandic discovery cohort (OR = 1.51, $P = 3.99 \times 10^{-10}$). This finding was replicated in cohorts from the United Kingdom and the Netherlands, showing an improved association and significantly increased risk of hand OA (OR = 1.46, $P = 1.10 \times 10^{-11}$). In silico replication found a significant association with this variant for knee OA, but not hip OA. Interestingly, this was a protective effect (OR = 0.95, $P = 0.044$), the opposite of the effect is seen on hand OA. The authors were surprised at this finding as the literature has previously suggested a close relationship between hand and knee OA[67] (Table 24.2).

4 FUNCTIONAL AND MOLECULAR PATHOLOGY

4.1 Comparison Between OA Genes and Monogenic Syndromes

A comparison between Tables 24.1 and 24.2 shows that monogenic disorders that result in early OA derive from defects in ECM-related molecules and cartilage metabolism, highlighting the importance of ECM in cartilage in both human skeletal development and OA. Yet, these molecules appear, according to the results from genetic association studies of OA, not to play a major role in terms of the genetic contribution toward the common form OA.

A consideration necessary to account for this discordance is to define what is OA.

OA is a disease of the whole joint, including bone, tendons, and synovium, not just cartilage. The involvement of these other tissues, in addition to cartilage, is essential to the development of the disease (Fig. 24.1).

Moreover, as mentioned, OA is not a single disease but is a group of disorders with a common outcome of joint failure. There is evidence that the various inter and intraarticular patterns of OA have distinctive risk factor profiles[74] and may result in different disease course and in theory might even respond differently to various treatments. Defining OA subphenotypes becomes thus necessary to understand the molecular pathogenesis of the various forms of OA and its genetic risk factors.

Finally, the clinical presentation of OA is not joint damage or skeletal deformity, but pain and disability. What leads a patient to seek medical help for OA is pain. Because cartilage is aneural and the causes of pain in OA are unlikely to be found in cartilage biology, a greater understanding of the relationship between the clinical manifestation of OA and cartilage damage is required.

4.2 A Disease of the Whole Joint

Pathology in OA is not restricted to cartilage, and differences in bone architecture are also observed.[75] Late changes, such as the presence of cysts and osteophytes, are hallmarks of OA. Furthermore, there is accumulating evidence that synovial inflammation is implicated in many of the signs and symptoms of OA, including joint swelling and effusion.[76] Histologically, the OA synovium shows hyperplasia with an increased number of lining cells and a mixed inflammatory infiltrate consisting mainly of macrophages.[77]

Synovitis is also likely to contribute to disease progression.[12] The overproduction of cytokines and growth factors from the inflamed synovium may play a role in the pathophysiology of OA. The low-grade synovitis in OA is itself cytokine-driven, although the levels of proinflammatory cytokines are lower than in rheumatoid arthritis (RA).[12,78] In particular, tumor necrosis factor-alpha (TNF-α) and interleukin (IL-1) have been suggested to be key factors in OA pathogenesis, both in synovial inflammation and in activation of chondrocytes.[12] These cytokines can stimulate their own production, and they can induce synovial cells to produce IL-6, IL-8, and leukocyte inhibitory factor, as well as protease and prostaglandin.[79] The presence of activated T cells and Th1 cytokine transcripts in chronic joint lesions of patients with OA indicates that T cells contribute to chronic inflammation in a large proportion of these patients.[80]

Moreover, the decreased osteophyte formation and synovial fibrosis following in vivo depletion of synovial macrophages in mice with collagenase-induced OA indicates that synovial lining macrophages mediate both osteophyte formation and synovial fibrosis.[81] The decreased formation of osteophytes following intraarticular injection of transforming growth factor TGFβ in these mice shows that the osteophyte-inductive effects of TGFβ are mediated by macrophages.[82]

Chondrocytes themselves are protagonists in this detrimental cascade of change, not only as the target of external stimuli, but as the cellular source of cytokines, chemokines, proteases, and inflammatory mediators that promote the deterioration of articular cartilage.[12] Pathogenic molecules produced by OA chondrocytes include MMPs, TNF-α, IL-1, IL-6, IL-8, nitric oxide, prostaglandins, and leukotrienes.[78] IL-1 stimulates the expression of MMPs and aggrecanases (ADAMTSs), which induce cartilage degradation. IL-1 is also capable of reducing the production of cartilage-specific macromolecules, including type II collagen. Conversely, TGFβ counteracts most of the IL-1 deleterious effects and contributes to cartilage homeostasis.[83]

Increasing evidence supports the hypothesis that tissue restoration and remodeling in OA are regulated by signaling pathways that are also involved in the development of cartilage and bone.[84] In particular, TGFβ, bone morphogenetic protein (BMP), and wingless-type (Wnt) signaling have been studied in OA and contribute to genetic susceptibility to OA.

Periarticular bone is not structurally normal in OA.[12,85] It has increased turnover, decreased bone mineral content and stiffness, and decreased trabecular numbers. Individuals with OA can exhibit striking increases in bone mass for affected sites, such as the knee and hip, as well as nonsynovial sites, such as the lumbar spine.[85] This appears to be due to abnormal osteoblast function, particularly in the subchondral bone tissue, which appears to be a response to altered local signals. It has been hypothesized that enhanced bone remodeling may be the initiating event that triggers cartilage damage.[86]

Hence, what are the molecular pathways that are actually involved in genetic susceptibility to OA?

4.3 Molecular Pathways Implicated in OA Genetic Risk

4.3.1 Functional Validation of GWAS Hits

A few of the OA susceptibility loci described in Table 24.2 have been the subject of functional validation studies, including rs2615977 in the COL11A1 gene, rs6976 in the GLT8D1/GNL3 region, rs3815148 between the HBP1 and COG5 genes, rs11177 in the GNL3 gene, and rs6094710 in the *NCOA3* gene. Gene expression or protein levels were found to be significantly different for these when comparing OA and non-OA human joint tissues.[87]

Silencing of the *DOT1L* gene inhibited chondrogenesis in human chondrocytes in vitro and this was further confirmed in vivo in murine models of OA where DOT1L was seen to play an important role in cartilage development. *IGFBP3* knockdown in chondrocytes prevented hypertrophy, a change associated with OA pathogenesis.[61] Similarly, overexpression of *IGFBP3* led to changes to cartilage consistent with those seen in OA pathogenesis.[61]

Some of the other pathways that have arisen from candidate and GWAS studies are discussed later.

4.3.2 Bone Morphogenetic Proteins

BMPs are members of the TGF-β superfamily of signal molecules that mediate many diverse biological processes. BMPs trigger cellular responses mainly through the Smad pathway, although the signal molecules can also activate the mitogen-activated protein (MAP) kinase pathway.[88] In model organisms a remarkable array of long distance modular regulatory elements surrounding the genes which encode BMPs has been identified.[88,89] These sequences correspond to individual "anatomy" elements that help control the size, shape, and number of individual bones and joints.[89] Genes in this pathway have been reproducibly associated with risk of OA.

4.3.2.1 GDF5

Regulatory elements from the growth and differentiation factor 5 gene (*GDF5*), also a BMP, can be used to inactivate other genes in joints, making it possible to identify genes and signals required for maintenance or repair of articular cartilage.[90]

An association between hip and knee OA and a single SNP (rs143383, T/C) located in the 5′-UTR of *GDF5* was reported in Japanese and in Chinese case-control cohorts.[53] The major T allele of the SNP was common in Asian populations, with frequencies >70% in controls,

and was at an elevated frequency in OA cases, with ORs ranging from 1.30 to 1.79 for knee and hip cases.[54] In vitro cell transfection studies revealed that the T allele mediated a moderate but significant reduction in the activity of the *GDF5* promoter. The same T allele was found at a higher frequency in hip and knee OA cases from Spain and the United Kingdom, relative to controls with a very modest OR of 1.10. A smaller effect has also been observed in other European samples in additional independent studies.[67,91] A subsequent large-scale metaanalysis found an OR of 1.17 (95% CI 1.12–1.23; $P = 6.2 \times 10^{-11}$) for knee OA for the T allele grouping both Asians and Caucasians, with no significant between-study heterogeneity across sample sets.[57]

However, the association of this gene variant with hip OA has been much more controversial.[91] Evangelou et al.[91] metaanalyzed the rs143383 in a large number of cases and controls, and is found by random-effects analysis only by a modest association (OR 1.16, 95% CI 1.03–1.31; $P = 0.016$) and very large interstudy heterogeneity ($I^2 = 75\%$). More recently, the role of this *GDF5* promoter polymorphism has been investigated in severe hip and knee OA by stratifying cases according to their generalized OA (nodal) status.[92] An association similar to knee OA was seen between rs143383 and hip OA, but only for the generalized nodal OA phenotype (OR = 1.27 95% CI 1.11–1.45; $P < 6 \times 10^{-4}$). Among severe hip OA patients without the generalized OA phenotype, this variant showed no evidence of association with hip OA (OR = 1.02 95% CI 0.92–1.14; $P < 0.56$). Based on these and other data, the authors concluded that generalized and nongeneralized forms of severe symptomatic hip OA have different pathogenesis, and this heterogeneity is in part genetically determined.

Other genes in the BMP pathway that have been implicated in genetic risk of OA include *ASPN*[93] and *SMAD3*.[94] Indirectly related to the BMP pathway is the genetic association reported between the deiodinase, iodothyronine, type II gene (*DIO2*) and OA susceptibility.[95] *DIO2* encodes an intracellular enzyme in the thyroid pathway that plays an essential role in the control of chondrocyte proliferation and differentiation, inhibiting BMP-2-induced growth.

4.3.3 Inflammation and Immune Response

Inflammatory changes can occur in synovium that likely contribute to the overall effects observed in OA, implying that inflammatory and degradative activities of synoviocytes are important in the pathogenesis of OA.[12] Hence, enzymes produced by the synovial lining cells can directly degrade matrix molecules.[96] This low-grade OA synovitis is cytokine-driven as involved in inflammation. Although, a number of variants in genes encoding for cytokines (in particular IL-1, IL-6, and IL-10) have been implicated in OA (for review, see Ref.[13]), some have been proven not to be reproducible when tested in large scale metaanalyses.[97] Although a relatively small study found variants in the IL1 receptor antagonist (ILRN) to be strongly associated with disease severity[98] (Table 24.1), and a role of this haplotype was confirmed in a larger independent replication.[98] On the other hand, studies in the same large metaanalysis concluded that the common polymorphisms in the IL1 gene are not associated with knee or hip OA.[97]

IL-1 and TNF-α also increase the synthesis of prostaglandin E2 (PGE_2). A pooled GWAS identified a variant mapping between *PTGS2* (the gene encoding the COX-2 enzyme) and *PLA2G4A* as significantly associated with the risk of knee OA in women from five different Caucasian cohorts.[66]

Another important class of molecules involved in inflammation and immune response are those mapping the major histocompatibility complex (MHC). A large-scale Japanese GWAS recently identified two SNPs to be strongly associated with knee OA.[56] One of the markers mapped to the MHC class II gene *DQB1* and was genome-wide significant in Japanese samples (Table 24.2), although an effect in the opposite direction was seen in Caucasians.[70] The other marker mapped to intron 1 of the butyrophilin-like 2 gene (*BTNL2*) gene, which regulates T-cell activation.[56] In the initial report, the SNP rs10947262 was significantly associated in both Asians and Caucasians and achieved genome wide significance overall. The presence of activated T cells and Th1 cytokine transcripts in chronic joint lesions of patients with OA indicates that T cells contribute to the chronic, low-grade inflammation in OA. T cells and chondrocytes have been reported to interact through cell surface molecules, such as MHC (HLA), CD4 or CD8 in OA and proliferative responses of peripheral blood T cells from patients with OA are substantially higher than those of control T cells from normal donors.[82] These and other observations support the hypothesis of a role for HLA in susceptibility to OA in agreement with the results from the Japanese GWAS. More recently, we investigated which specific HLA class II antigen presenting haplotypes were in LD with the Japanese GWAS marker. We found that the SNPs associated with knee OA in Japanese patients tags the haplotype DRB1*1502 DQB1*0601, which has a frequency around 8.2% in Japanese. According to the Japanese GWAS, this should be protective for knee OA. This haplotype, however, is much rarer both in Chinese (2.3%) and European (0.8%) samples; and furthermore, it is not in LD with the same SNPs.[70] It can be concluded that there is strong evidence for a role in susceptibility to OA from the HLA class II region from Japanese studies, yet a proper study to investigate HLA in Caucasian and Chinese samples is necessary.

Other genes related to inflammation that have been tested for association with OA include the gene encoding the c-reactive protein (CRP), which has been recently

shown to have no effect on knee or hip OA in a large study,[99] and the TNF-α gene, which failed to show an association with knee OA in a Turkish study.[100]

4.4 Wnt Signaling

The Wnt signaling cascades have essential roles in development, growth, and homeostasis of joints and the skeleton.[101] As discussed earlier, mutations in the *WISP3* gene result in pseudorheumatoid dysplasia. Several studies have explored the relationship between OA and two polymorphisms in the frizzled-related protein (*FRZB)* gene: the Arg200Trp and Arg324Gly variants (SNP IDs rs7775 and rs288326), with some studies finding an association with hip OA[102,103] and knee OA.[104] Additional genetic evidence of a role for Wnt signaling in susceptibility to OA came from a GWAS that demonstrated a link between *DOT1L* and radiographic joint-space width, used as a proxy for cartilage thickness and hip OA, in a population-based study.[62,74] *DOT1L* encodes histone H3-K79 methyltransferase, a protein that has been linked to variety of cellular pathways but seems to be active mainly in association with TCF transcription factors of the canonical Wnt cascades[101] Together, these observations identify the Wnt pathway as an attractive target for therapeutic intervention for OA ; however, the complexity of the Wnt signaling cascades and the potential secondary effects of drug interventions targeting them highlight the need for further research.[101]

4.5 Pain in OA and Its Genetic Contribution

Relief from severe chronic OA pain remains an unmet medical need and a major reason for seeking surgical intervention for OA. There is extensive literature reporting discordance between the presence and severity of symptoms and the degree of radiographic structural OA.[105] The relationship between radiographic changes of OA and pain experienced by a patient is far from perfect and remains weak once the influence of psychological and social factors and comorbidity have been taken into account.[106] The mechanisms involved in pain due to OA are only poorly understood and the molecular etiology of pain in OA is an understudied field. To date only candidate gene studies have been carried out to identify genetic factors contributing genetic susceptibility to OA pain.[107]

5 DIAGNOSTIC ASPECTS

The advent of GWAS has accelerated the discovery of novel genetic markers that are associated with risk for many complex diseases, not just OA.[65] It is expected that in the next 5 years whole genome sequencing will facilitate the discovery of novel SNPs, as well as of rare variants, copy number variations, deletions/insertions, structural variations (e.g., inversions), and epigenetic effects that influence the regulation of gene expression. These developments are fuelling interest in the translation of this basic knowledge to health care practice. Knowledge about genetic risk factors may be used to target diagnostic, preventive, and therapeutic interventions for complex disorders based on a person's genetic risk, or to complement existing risk models based on classical nongenetic factors.

Risk prediction of knee OA has been established using conventional risk factors, such as age, gender, family history, body mass index, occupational risk, and joint injury,[9] setting the basis on which to improve risk prediction with genetic information.

The increased risk for OA conferred by carrying a predisposing genetic variant at any of the identified loci seems to be quite modest; most variants have only small effects on risk. Risk prediction models are used in research and clinical settings to classify individuals into homogeneous groups; for example, for randomization in clinical trials and targeting preventive or therapeutic interventions.[107] Risk prediction models are statistical algorithms, which can be simple genetic risk scores (e.g., risk allele counts), or be based on regression analyses (e.g., weighted risk scores or predicted risks) or on more complex analytic approaches, such as support vector machine learning or classification trees.[108] So far, most genetic prediction studies have shown that the predictive performance of genetic risk models is poor, with some exceptions, such as those for age-related macular degeneration, hypertriglyceridemia, and Crohn's disease.[109,110] This poor performance is most likely due to the low number of variants that have been definitely linked to any phenotype to date. Therefore, the more relevant loci that are found—even of small effect—the better the predictions should be for both high and low susceptibility to OA.

Kerkhof and coworkers developed a prognostic model for incident knee OA in a general population and determined the value of different risk factors, including genetic factors, to prediction. They found that there was moderate predictive value for incident knee OA based on the genetic score alone in subjects aged <65 years (AUC 0.65), but it was only 0.55 for subjects aged ≥65 years[111] suggesting that the genetic risk factors identified so far play a more relevant role in risk of OA for younger onset. At the same time, genetic risk factors appear to have the same influence for both idiopathic and posttraumatic OA.[112] Thus, although the predictive value of the variants identified to date is rather low and not clinically useful yet, it is likely to be applicable to predict risk of OA in the presence of knee injuries in younger subjects.

6 TREATMENT

While many disease-modifying therapies exist for the more aggressive and inflammatory forms of arthritis, such as rheumatoid arthritis, the options available for treating OA patients are limited.[12] No disease-modifying drugs for OA are approved by the Food and Drug Administration (FDA) or the European Medicines Agency (EMEA). In the absence of disease modifying drugs, clinical recommendations for treatment available for each level of OA severity from mildest to most severe are: (1) mildest = non pharmacological conservative intervention, such as exercise, weight loss, footwear; (2) mild = further nonpharmacologic management, such as physiotherapy and braces in addition to simple analgesics, such as paracetamol; (3) severe = pharmacologic management, nonsteroid antiinflammatory drugs (NSAIDs), opioids, intraarticular corticosteroids, intraarticular hyaluronates; and (4) end-stage OA = osteotomy, unicompartmental joint replacement, and TJR.[113]

It should be noted that the available options for pharmacotherapy, which aim to reduce the symptoms of OA, do so only with limited efficacy and leave the patient with a considerable burden of pain.[113]

Some studies have suggested that glucosamine sulfate, chondroitin sulfate, sodium hyaluronan, doxycycline, MMP inhibitors, bisphosphonates, calcitonin, diacerein, and avocado-soybean unsaponifiables may modify disease progression.[78] Nevertheless, structure-modifying efficacy has not been convincingly demonstrated for any existing pharmacologic agents and the difference between these proposed drugs and placebo is very small and extremely difficult to detect.[113]

6.1 Surgical Options

TJR is a frequently performed and effective procedure that relieves pain and improves functional status in patients with end-stage knee or hip OA.[114]

There are no generally accepted criteria for joint replacement surgery in patients with OA.[114] Typically, the diagnosis of OA is made on the basis of clinical examination and plain radiographs. However, as it is well recognized that radiographic severity is only loosely correlated with symptom severity and functional limitation,[105] the critical questions that drive recommendations for surgery are whether the patient has contraindications, and whether the patient's functional limitations are severe enough to warrant surgery, although the level of pain needed to indicate when surgery is appropriate is unclear, given the lack of standardized guidelines. In terms of outcomes, results from population-based studies indicate that approximately 80%–90% of patients having a TJR will have improvement in function and near complete relief of pain, and will be satisfied with the results of these surgeries.[4] Importantly, it has been shown that more severe radiographic damage presurgery correlates with lower pain and less disability post-surgery, indicating that the causes of severe chronic pain in patients with mild radiographic OA are not fully addressed by TJR surgery.

The major concern in long-term outcome of TJR is survival of the prosthesis.[114] The only population-based data available suggest over a follow-up of up to 4 years that the rate of failure of THR leading to revision is approximately 4% over 4 years, that is, 1% per year.[114] Wear debris, primarily generated from the prosthetic joint articular surface, remains the major factor limiting the survival of joint implants by contributing to osteolysis and aseptic loosening after THR.

7 CONCLUSIONS

Genetic discoveries have shaped our knowledge concerning the etiologic pathways and mechanisms implicated in the genetic architecture of OA, and have revealed the complexity underlying the genetic susceptibility to OA.

Although the study of monogenic diseases that resemble OA or result in early onset OA has greatly increased our knowledge of cartilage biology, it has not helped us understand the molecular genetic etiology of the complex, most prevalent form of OA. There are some shared points between these different types of studies. The most prevalent complex form of OA seems to be strongly influenced by molecules related to the BMP pathway, which is central to skeletal development.

On the other hand, genetic studies on OA and other complex diseases have highlighted the need for replication of findings, thus reducing the false-positive signals and supporting the need for collaborative efforts. GWAS studies have revealed considerable information about the genetic architecture of OA,[72] and we now know that sample sizes in the excess of 7000 cases and 7000 controls are needed to detect common variants as genome wide significant for this disease. Current evidence is that OA is a polygenic and complex disorder; contributed by many risk alleles, each with a small effect.[72] The GWAS and large candidate studies of OA have so far indicated BMPs (involved in skeletal development) and possibly nociception as etiological pathways in the late onset of OA, as it occurs in the general population. During the coming few years, additional genetic and functional studies will offer a more complete picture of the genetic contribution to OA and the underlying molecular pathways. Whole genome sequencing has become a reality, and it is expected that the first studies on complex diseases have already been published. Although, there are substantial bioinformatic and analytical challenges

ahead, it is to be expected that these novel technologies will reveal the role of many novel variants hopefully revealing molecular targets for novel therapies and making improved risk prediction a reality. If this promise is delivered, it will improve our ability to diagnose and treat OA, which represents a major healthcare burden.[115]

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CHAPTER

25

Genetics of Paget's Disease of Bone

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1 CLINICAL FEATURES

Paget's disease of bone (PDB) is characterized by focal abnormalities of increased and disorganized bone turnover, which preferentially target the axial skeleton.[1] The risk of developing PDB is strongly age dependent; it rarely presents clinically before the age of 50 years but increases in prevalence thereafter to affect up to 8% of men and 5% of women by the age of 80 in the United Kingdom. Bone resorption is markedly increased within Pagetic lesions, and this is accompanied by other abnormalities, such as marrow fibrosis, increased vascularity, and accelerated bone formation with the production of woven bone, which has impaired mechanical strength. This can lead to the development of bone deformity, especially when the disease affects weight-bearing bones of the lower limbs, or pathological fractures that can occur as the result of minimal or no trauma. The increased bone formation in PDB causes affected bones to enlarge, and this can lead to complications, such as spinal stenosis, deafness, and other nerve compression syndromes. Osteoarthritis is a common complication of PDB. The mechanism is incompletely understood, but may be due to abnormal mechanical stresses placed on the neighboring joints by bones that are deformed or impaired ability of the articular cartilage to absorb impact loading due to osteosclerosis of subchondral bone. High-output cardiac failure is a rare complication of PDB due to increased blood flow through affected bone in patients with extensive disease. Patients with PDB can also develop hypercalcemia when immobilized, due to increased release of calcium from the skeleton, but this is uncommon. There is some evidence that PDB patients have reduced survival when compared with age-matched controls due to an increased risk of cardiovascular disease.[2] Although the underlying mechanisms of cardiovascular disease are not well understood, PDB can be associated with vascular calcification, which could provide a potential explanation.[3] Rarely, PDB may be complicated by the development of osteosarcoma, which is a serious and often fatal complication of the disease.

It has been estimated that only 7%–14% of patients with PDB come to medical attention,[2,4] but symptoms and complications are common in those that do present clinically. In a recent survey, the most common presenting symptom was bone pain (73.8%), followed by deformity (18.1%), deafness (7.9%), and pathological fracture (5.7%). Epidemiological studies[2] have shown that patients with PDB are at an increased risk of several complications as compared with controls as summarized in Table 25.1.

There are marked ethnic differences in susceptibility to PDB.[5] The disease predominantly affects Caucasians, and the United Kingdom has the highest incidence of the disease worldwide, where about 1%–2% of people above the age of 55 are affected. Paget's is also common in Spain, Italy, and France and in people of European descent who have migrated to Australia, Canada, New Zealand, South Africa, and the USA.[5] Conversely, PDB is uncommon in Scandinavia and is rare in the Indian subcontinent, China, Japan, and other countries in the Far East.[5] These observations, when combined with archaeological studies of skeletal remains[6] are consistent with a model whereby PDB originated in the United Kingdom many centuries ago as the result of genetic mutations and spread to other parts of the world as the result of migration and admixture.

A striking feature of PDB is its unusual distribution. It predominantly affects the axial skeleton, with a pattern of involvement that is usually asymmetric. The reasons for this are unclear, but suggested explanations include targeting of the disease to limbs or bones that are subject to repetitive mechanical loading, and somatic mutations of the affected bones (discussed in more detail later).

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TABLE 25.1 Complications of Paget's Disease of Bone (PDB)

Complications	Frequencies (%)	Relative risks (95% CI)
Fracture	2.8	1.2 (1.0–1.5)
Hearing loss	2.4	1.6 (1.3–1.9)
Back pain	10.2	2.1 (1.9–2.3)
Osteoarthritis	5.6	1.7 (1.5–1.9)
Hip arthroplasty	1.5	3.1 (2.4–4.1)
Bone neoplasm	0.1	Infinity (5.0–infinity)
Heart failure	4.6	1.2 (1.1–1.4)

The annual incidence of various complications of PDB over a 3-year follow-up period in a cohort of 2465 patients with PDB from the General Practice Research Database in the United Kingdom is shown. The relative risk of each complication was assessed by comparing the cases with three age-matched, randomly selected controls from the same database.
From van Staa TP, Selby P, Leufkens HG, Lyles K, Sprafka JM, Cooper C. Incidence and natural history of Paget's disease of bone in England and Wales. J Bone Miner Res *2002;**17**(3):465–71.*

2 GENETIC ARCHITECTURE OF PAGET'S DISEASE

Several lines of evidence indicate that genetic factors play a central role in PDB. Familial clustering is common and it has been reported that the disease occurs about 7 times more commonly in first-degree relatives of affected individuals as compared with controls.[7,8] It is of interest that the proportion of patients who have a family history varies markedly between countries, ranging from about 5% in Holland to 50% in the French–Canadian population.[9,10] In the United Kingdom and Italy, between 12% and 15% of affected individuals have a family history.[11,12] This is likely to be due in part to a founder effect, but it's possible that other factors, such as environmental triggers or variations in ascertainment, may differ between countries. The marked ethnic variations in disease prevalence favor a genetic etiology, as they persist in subjects who have migrated from high-prevalence regions, such as the United Kingdom and Europe, to countries, such as Australia and New Zealand where PDB is rare in the indigenous population. Many families have been reported in which classical PDB is inherited in an autosomal dominant manner, which increases in penetrance with age.[10,13,14] In addition to classical PDB, several rare disorders have been described with clinical features overlapping with PDB where the disease is inherited in a Mendelian manner with an early age of onset. These syndromes are dealt with in more detail elsewhere in this book, but will be discussed briefly here.

Familial expansile osteolysis (FEO), expansile skeletal hyperphosphatasia, and early-onset familial PDB are inherited in an autosomal dominant manner. They are allelic disorders with overlapping clinical features that present in childhood or early adulthood, which are characterized by early deafness, tooth loss, and the development of osteolytic expansile bone lesions.[15]

The syndrome of inclusion body myopathy, PDB, and frontotemporal dementia (IBMPFD) is a rare condition, which is inherited in an autosomal dominant manner[16] and is caused by mutations in the *VCP* gene.[17] The most prominent feature of this syndrome is myopathy, which presents in the third or fourth decade, but about 40% of patients develop PDB typically in the fourth decade and about 40% develop dementia in the fifth or sixth decade. A familial form of PDB with myopathy and neurodegenerative disease has also been described in association with mutations in the heterogeneous nuclear ribonucleoprotein A2B1 and A1 genes (*HNRNPA2B1* and HNRNPA1), which encode prion-like domain–containing proteins involved in RNA binding and formation of RNA granules.[18] The disease-causing mutations have been shown to promote formation of abnormal protein aggregates, which cause cellular toxicity in neurones and muscle.[18] Juvenile Paget's disease (JPD) is inherited in an autosomal recessive manner. It is usually caused by mutations in the TNF receptor superfamily 11B (*TNFRSF11B*) gene, which encodes osteoprotegerin,[19] but a case has been described in which a 15-bp insertion mutation of the *TNFRSF11A* gene was responsible.[20] Juvenile PDB presents in childhood with bone pain, deformity, and fractures during childhood in association with a generalized increase in bone turnover. Radiographs show evidence of bone enlargement and deformity with mixed lytic and sclerotic lesions, reminiscent of classical PDB. Another syndrome of severe familial PDB associated with giant-cell tumor has been described in patients from the Campagnia region of Italy.[21] The familial pattern of disease coupled with the geographical distribution suggested a founder effect, and it has recently been reported that this syndrome is due to mutations in the *ZNF678* gene.[22]

3 ENVIRONMENTAL FACTORS

Although there is compelling evidence that genetic factors play a central role in PDB, environmental exposures also appear to exert an important influence on the development and severity of the disease. Evidence of this comes from observations made in epidemiological studies, which have shown significant decreases in disease prevalence and severity in many countries over the past 25 years.[5] It is of interest, however, that in some countries, such as Italy and some regions of Spain, no major changes in prevalence or severity of PDB have been observed.[23,24] The most widely studied environmental trigger is viral infection, focusing especially on the possibility that PDB may be caused by a persistent "slow" virus infection with one of the paramyxoviruses.[25] Several attempts have been made to detect evidence

TABLE 25.2 Genes That Cause PDB and Related Syndromes

Genes	Gene product	Loci	Gene functions	Clinical syndromes
CSF1	M-CSF	1p13	Enhances osteoclast differentiation	Classical PDB
DCSTAMP	Dendritic cell-specific transmembrane protein	8q22	Essential for fusion of osteoclast precursors to form multinucleated osteoclasts	Classical PDB
OPTN	Optineurin	10p13	Inhibits NFκB signaling and stimulates osteoclast differentiation	Classical PDB
HNRNPA2B1	Heterogeneous nuclear ribonucleoprotein A2B1	7p15	Regulates RNA processing and stability	Myopathy, neurological disease PDB
HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1	12q13	Regulates RNA processing and stability	Myopathy, neurological disease PDB
PML	Promyelocytic leukemia protein	15q24	Regulates TGFβ signaling and apoptosis	Classical PDB
RIN3	Rab and Ras interactor 3	14q32	Function in bone unclear	Classical PDB
SQSTM1	p62	5q35	Stimulates NFκB signaling and osteoclast differentiation	Classical PDB, familial PDB
TNFRSF11A	RANK	18q21	Activates NFκB signaling and osteoclast differentiation	Classical PDB, FEO, EoPDB, ESH
TNFRSF11B	OPG	8q24	Inhibits RANKL induced NFκB signaling and osteoclast differentiation	Juvenile PDB
VCP	p97	9q21	Regulates NFκB signaling	Inclusion body myopathy, PDB, dementia

EoPDB, Early-onset PDB; ESH, expansile skeletal hyperphosphatasia; FEO, familial expansile osteolysis; M-CSF, macrophage colony-stimulating factor; TGFβ, transforming growth factor beta.

of persistent viral infection in cells and tissues from affected patients, but the results have been conflicting.[26,27] Other suggested, but as yet unconfirmed, triggers for the disease include dietary calcium deficiency,[28] vitamin D deficiency,[29] exposure to environmental toxins,[30] and excessive mechanical loading of affected bones.[31]

4 MOLECULAR GENETICS

Major advances have been made over recent years in understanding the molecular genetic basis of PDB through linkage studies, genome-wide association studies (GWAS), and genomewide sequencing studies. This has resulted in the identification of several loci and genes that predispose to PDB as summarized in Table 25.2. It is relevant that many of the genes that have been implicated in the pathogenesis of PDB and related disorders affect osteoclast differentiation or function (Figs. 25.1 and 25.2). These are discussed individually in more detail here.

4.1 *CSF1*

One of the strongest hits to emerge from a GWAS of PDB was on chromosome 1p13 approximately 87-kb upstream: the colony-stimulating factor 1 (*CSF1*) gene.[32,33] The *CSF1* gene encodes macrophage colony-stimulating factor (M-CSF), which is a strong candidate gene for PDB because it plays a critical role in regulating osteoclast and macrophage differentiation and because serum M-CSF levels have been reported to be elevated in PDB patients as compared with controls.[34] The predisposing variants cluster in a region that is 87-kb upstream of *CSF1*, which is marked by acetylation of lysine 27 residues of histone 3 (H3K27ac). This relevant, as H3K27ac marks are characteristic of active enhancer elements,[35] suggesting that the variants in this region might predispose an individual to PDB by regulating *CSF1* expression. While previous studies have shown that serum M-CSF levels are higher in patients with PDB as compared with controls,[36] further work will be required to determine if the variants at 1p13 predispose an individual to PDB by upregulating M-CSF production.

4.2 *OPTN*

The *OPTN* gene, which encodes optineurin, lies on chromosome 10p13, which was originally identified as a susceptibility locus for familial PDB by linkage analysis.[37] Subsequently a GWAS involving patients with both familial and sporadic PDB showed evidence of a strong association with the disease and several single-nucleotide polymorphisms (SNPs) within the *OPTN* gene.[33] The

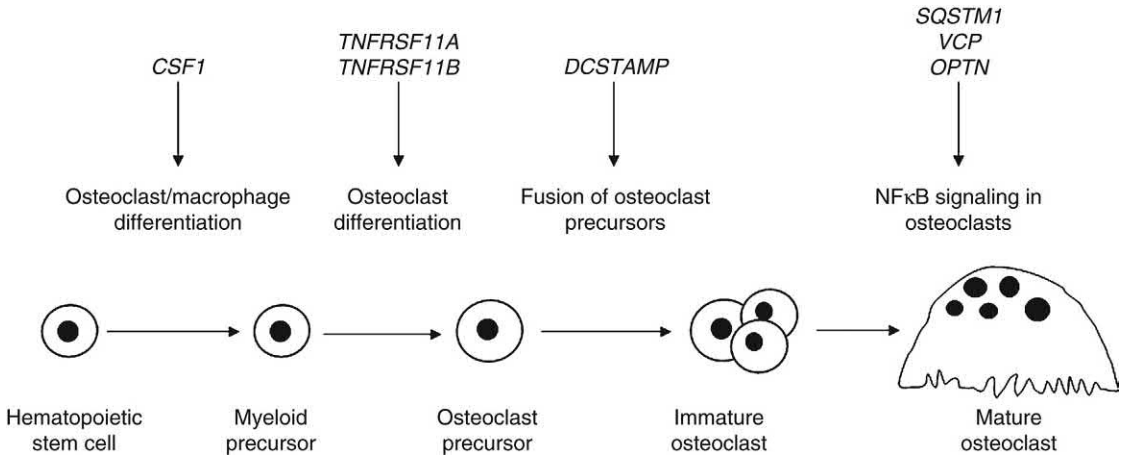

FIGURE 25.1 Role of PDB susceptibility genes in osteoclast function. Many of the genes implicated in PDB regulate osteoclast differentiation and activity. Genetic variants in the colony-stimulating factor 1 (*CSF1*), TNF receptor superfamily 11A (*TNFRSF11A*) and 11B (*TNFRSF11B*), and dendritic cell–specific transmembrane protein (*DCSTAMP*) genes most likely predispose an individual to PDB by affecting differentiation of osteoclasts at the points indicated, whereas variations in the Sequestosome 1 gene (*SQSTM1*), valosin-containing protein (*VCP*), and optineurin (*OPTN*) genes predispose an individual to PDB by influencing NFκB and other signaling pathways in osteoclasts or their precursors.

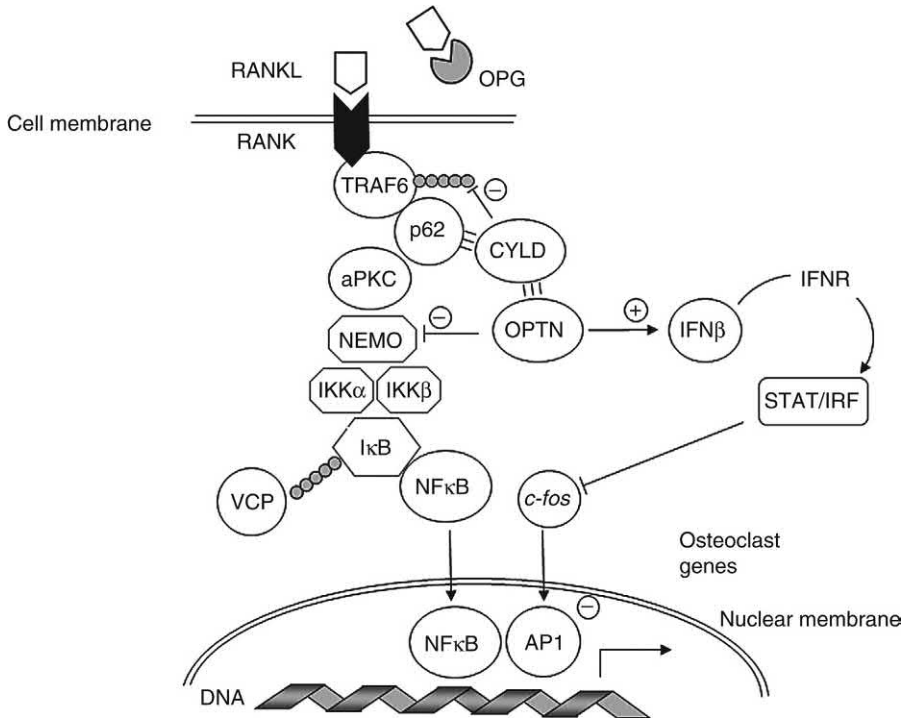

FIGURE 25.2 Molecular mechanisms of osteoclast activation. The figure illustrates selected aspects of osteoclast activation with a particular focus on genes implicated in the pathogenesis of Paget's disease. The binding of receptor activator of NFκB ligand (RANKL) to receptor activator of NFκB (RANK) initiates signals that lead to osteoclast activation, and this is inhibited by osteoprotegerin (OPG), a decoy receptor for RANKL. When RANKL engages with RANK, several proteins are recruited to the intracellular domain of the RANK receptor, including p62, TNF-associated factor 6 (TRAF6), and atypical protein kinase C (aPKC). The p62 protein recruits cylindromatosis (turban tumor syndrome) (CYLD) to the intracellular protein complex where it deubiquitinates TRAF6, thereby inhibiting NFκB signaling. The p62 protein is also involved in regulating NFκB signaling through an interaction with aPKC, which activates the NFκB essential modulator–IκB kinase (NEMO–IKK) complex, leading to phosphorylation of IκB. The phosphorylated IκB is ubiquitinated and targeted for proteosomal degradation leaving NFκB free to translocate to the nucleus and activate osteoclast-specific gene expression. It is thought that VCP may also influence degradation of IκB. OPTN negatively regulates NFκB signaling through an effect on NEMO and also binds to CYLD, which most likely inhibits NFκB signaling by deubiquitinating TRAF6. In addition, OPTN stimulates production of interferon beta production (IFNβ), which activates of its receptor (IFNR) and downstream signaling molecules, such as signal transducer and activator of transcription (STAT) and interferon-regulating factor (IRF), to inhibit *c-fos*, a component of the activating protein 1 (AP1) transcription factor, which stimulates osteoclast differentiation.

10p13 locus is bounded by two recombination hotspots, containing *OPTN* and no other known genes. Missense mutations in *OPTN* have been described in patients with glaucoma and in amyotrophic lateral sclerosis. Functional studies have shown that the mutations that cause amyotrophic lateral sclerosis activate NFκB signaling in vitro and result in the accumulation of *OPTN*-containing cytoplasmic inclusions in neurons. The top hit single-nucleotide polymorphism identified by GWAS on 10p13 is a strong expression quantitative trait locus (eQTL) for *OPTN* expression, and levels of mRNA are lower in carriers of the variant that predispose to PBD.[33] In keeping with this, it has recently been shown that optineurin acts as a negative regulator of NFκB signaling in osteoclasts both directly and indirectly through an effect on interferon beta (IFNβ) signaling.[38] The data are consistent with a model whereby regulatory variants at the *OPTN* locus predispose an individual to PDB by reducing expression of optineurin, which in turn increases osteoclast differentiation and activity.

4.3 *SQSTM1*

Mutations in the Sequestosome 1 gene (*SQSTM1*) were identified as an important cause of classical PDB through linkage analysis and positional cloning studies in families of French–Canadian and British descent.[39,40] The first causal mutation was identified in the French–Canadian population as a proline-to-leucine change at position 392 of the gene product (P392L),[41] but subsequently this and other mutations clustering in the ubiquitin-associated (UBA) domain of *SQSTM1* were found in patients of British descent. A large number of *SQSTM1* mutations have now been identified in patients with PDB and most,[42] but not all,[43] of these are located within the UBA domain, impairing its ability to bind ubiquitin.

The *SQSTM1* gene encodes p62, which is an adaptor protein that plays an important role in regulating NFκB signaling by interacting with other proteins, including TNF-associated factor 6 (TRAF6), atypical protein kinase C (aPKC), and the deubiquitinating enzyme, cylindromatosis (turban tumor syndrome) (CYLD).[44] Mutations of *SQSTM1* occur in between 40% and 50% of patients with a family history of PDB, and between 5% and 10% of patients with sporadic disease.[45] It is thought that *SQSTM1* mutations lead to PDB by interfering with the ability of p62 to bind ubiquitin, leading to enhanced NFκB signaling and increased sensitivity of osteoclast precursors to receptor activator of NFκB ligand (RANKL) by mechanisms that are discussed later in this chapter.

4.4 *TNFRSF11A*

The *TNFRSF11A* gene encodes the receptor activator of NFκB (RANK), which is a member of the TNF receptor family that plays an essential role in osteoclast differentiation and bone resorption.[46] The *TNFRSF11A* gene was initially implicated in the pathogenesis of PDB by linkage analysis in patients with the severe PDB-like syndrome of FEO. These studies localized the causal gene for FEO to the *TNFRSF11A* locus on chromosome 18q21 in 1994,[47] and subsequently, some cases of familial PDB were found to be linked to the same region.[48] Mutation screening of *TNFRSF11A* resulted in the identification of an 18-bp duplication affecting the signal peptide of RANK as the cause of FEO and a duplication of 27 bp in the RANK signal peptide as the cause of early-onset familial PDB.[49] Subsequently, duplications of 12 and 15 bp involving the same region of RANK were found in patients with expansile skeletal hyperphosphatasia, a disorder with phenotypic similarities to FEO and in Juvenile PDB.[20,50,51] These mutations prevent cleavage of the RANK signal peptide, and this causes the mutated RANK molecules to accumulate in the Golgi apparatus.[52] The insertion mutations in RANK molecules were originally reported to activate NFκB signaling when overexpressed in human embryonic kidney (HEK) cells.[49] However more recent studies have shown that HEK cells that have been engineered to express the mutant RANK molecule at normal levels show no evidence of an increase in NFκB signaling and fail to initiate NFκB or ERK signaling in response to RANKL, indicating that they are loss-of-function mutations.[52] Paradoxically, however, expression of the RANK molecules bearing the insertion mutations stimulate osteoclast formation in vitro when expressed in osteoclast precursors by mechanisms that are incompletely understood.[52]

Mutations have not been detected in the exons or intron–exon boundaries of *TNFRSF11A* in classical PDB, but there is strong evidence that common genetic variants at the *TNFRFS11A* locus predispose an individual to classical PDB.[33] The mechanisms by which common genetic variants at this locus predispose an individual to PDB remain unclear; the predisposing variants identified by GWAS do not appear to influence RANK mRNA expression, at least as judged by expression quantitative trait locus analysis in leucocytes.[32] A common protein coding variant, V192A, within exon 6 of *TNFRFS11A* has been reported in one study to influence NFκB signaling in reporter assays,[53] but other investigators were unable to replicate this finding.[54] In view of this further research will be required to fully elucidate the mechanisms by which variants at the *TNFRSF11A* locus predispose an individual to PDB.

4.5 *TNFRSF11B*

The *TNFRSF11B* gene encodes osteoprotegerin (OPG), which plays a key role in regulating osteoclast

differentiation and function by blocking the stimulatory effects of RANKL on osteoclast differentiation.[55] Mutations affecting the *TNFRSF11B* gene were identified as a cause of JPD by Whyte et al. who described two native American patients with JPD that were homozygous for a 100-kb deletion of chromosome 8q24 involving the whole *TNFRSF11B* gene.[56] These subjects had undetectable circulating levels of OPG and markedly increased bone turnover. A subsequent study identified a homozygous 3-bp deletion in affected siblings from an Iraqi family, which resulted in loss of a conserved aspartate residue at codon 192.[57] Functional studies showed that the mutant form of OPG was unable to prevent osteoclastic resorption in a bone culture system, confirming that it was a loss-of-function mutation. Subsequently, several missense mutations of *TNFRSF11B* were described in JPD, most of which affect the cysteine-rich ligand-binding domain, which is involved in binding of OPG to RANKL.[19] Mutations of *TNFRSF11B* have so far not been detected in classical PDB and the 8q24 locus did not emerge as a susceptibility locus for PDB in a GWAS.[32] There is some evidence that common variants at the *TNFRSF11B* locus might predispose women to classical PDB, but not men,[58] but this remains to be confirmed by a large-scale study.

4.6 *VCP*

The *VCP* gene encodes valosin-containing protein, which is a member of the $AAA^{(+)}$ ATPase family of chaperone-like proteins that regulate numerous cellular processes, including chromatin organization, membrane fusion, ubiquitin-dependent protein degradation, and autophagy.[59] Mutations in *VCP* have been shown to be the cause of IBMPFD.[17] The causal gene was localized to 9p21 by a genome-wide linkage scan,[16] and subsequently positional cloning studies identified several missense mutations affecting the highly conserved CDC48 domain as the cause of the disease.[17] It is thought that the myopathic and neurological manifestations of IBMPFD are due to accumulation of abnormal protein aggregates in muscle and neurons; although the mechanisms by which *VCP* mutations cause bone disease are not well understood. Possible mechanisms include a modulatory effect on the NFκB signaling pathway, as VCP is known to play a role in the degradation of phosphorylated IκB[60] or through an effect on autophagy, which is impaired in the presence of *VCP* mutations.[61] There is some evidence that protein-coding mutations of VCP may rarely occur in patients with classical PDB who do not have other components of the IBMPFD syndrome.[62] There is no firm evidence that common variants at the VCP locus predispose an individual to PDB.

4.7 *HNRNPA2B1* and *HNRNPA1*

Mutations in two members of the hnRNP gene family have been identified as the cause of a multisystem proteinopathy syndrome characterized by myopathy, neurodegeneration, and PBD.[18] The *HNRNP* gene family encode multifunctional proteins involved in mRNA splicing, processing stability, and turnover.[63] The phenotype in one affected family consisted of autosomal dominant inheritance of cognitive impairment, myopathy, PDB, and motor neuron dysfunction. In this case the causal mutation was found to be an aspartic acid–to-valine substitution at codon 290 of the short isoform of the *HNRNPA2B1* gene (p.D290V). In another family with inheritance of myopathy and PDB, the mutation was identified as an aspartic acid–to-valine change at codon 262 (p.D262V) of the short isoform of the *HNRNPA2* gene. A third family was described in which p.D262V was associated with inheritance of amyotrophic lateral sclerosis in the absence of PDB or myopathy. The aspartic acid residue that was affected in both genes is highly conserved across species and is situated in a prion-like domain motif that is conserved in *HNRNPA2B1* and *HNRNPA2*, as well as other members of the *HNRNP* gene family. Bioinformatic analysis demonstrated that the causal mutations were predicted to enhance the likelihood of the proteins self-assemblage to form fibrils, and this was confirmed experimentally both in vitro and in vivo when the abnormal proteins were over expressed in *Drosophila*. Importantly, overexpression of the abnormal proteins also caused muscle degeneration in *Drosophila*, supporting the hypothesis that the protein aggregates were responsible for the muscle phenotype observed clinically. As is the case with VCP disease, the mechanisms underlying the pathogenesis of PDB in this syndrome are less clear and require further investigation.

4.8 *DCSTAMP*

The dendritic cell–specific transmembrane protein gene (*DCSTAMP*, formerly known as *TM7SF4*), located on chromosome 8q22 was identified as a possible candidate for PDB through a GWAS.[32] The top hit rs2458413 is located in an 18-kb linkage disequilibrium block, which encompasses the entire *DCSTAMP* gene. This gene is a strong functional candidate for PDB, as its product plays an essential role in the fusion of mononuclear osteoclast precursors to form multinucleated osteoclasts.[64] It is not yet clear if there is a specific ligand for DCSTAMP, but it has been recently shown that connective tissue growth factor (CTGF) physically interacts with DCSTAMP and upregulates DCSTAMP expression to enhance RANKL-induced osteoclast differentiation.[65] A rare coding variant causing a substitution of leucine to phenylalanine at position 397 (p.L397P),

has been described in a patient with PDB, but the clinical significance and functional effects of this variant have not yet been investigated.[66] Currently the mechanisms by which common genetic variants at the *DCSTAMP* locus predispose an individual to PDB are unclear.

4.9 *RIN3*

The Rab and Ras interactor 3 (*RIN3)* gene situated on chromosome 14q32 was implicated as a genetic regulator of susceptibility to PDB by an extended GWAS.[32] The top hit rs10498635 lies within the *RIN3* gene and is in linkage disequilibrium with a common missense variant within the coding region (p.R279C), which is strongly associated with PDB susceptibility. Sequencing of the *RIN3* gene identified multiple rare missense variants, which were enriched in PDB cases as compared with controls. Most of these clustered in the proline-rich domain of the *RIN3* protein, but one mutation was situated in the *Src* homology 2 (SH2) domain and another three were situated in the vacuolar sorting protein 9 (VSP9) domain. Bioinformatic analysis showed that the common variant p.R279C and four rare variants (p.A141V, p.R231C, p.P386S, and p.Y793H) were likely to be functional, but analysis of variants in the proline-rich domain was inconclusive due to the unstructured nature of this region of the protein. The RIN3 protein has been shown to be involved in vesicular trafficking and to interact with amphiphysin II, a protein involved in the regulation of endocytosis[67]; RIN3 has also been shown to participate in the internalization of receptor tyrosine kinase KIT in mast cells.[68] The functional role of RIN3 in bone is unclear, but it has been shown to be expressed in Pagetic osteoclasts, normal osteoclasts, bone marrow cells, macrophages, and at low levels in osteoblasts.[69] The mechanisms by which genetic variants in *RIN3* affect bone cell function and predispose an individual to PDB are unclear.

4.10 *PML*

The promyelocytic leukemia gene (*PML*), located on chromosome 15q24, was identified as a potential genetic marker for susceptibility to PDB by an extended GWAS.[32] The 15q24 locus contains two known genes, but the top hit rs5742915 lies within the *PML* gene and causes a phenylalanine-to-leucine amino acid change at codon 645 of the gene product (p.P645L). Recent deep-sequencing studies (Wani, Ralston, and Albagha, unpublished) suggest that the association is due to variants within the *PML* gene rather than other genes in this locus. The PML protein is involved in regulating cell growth, apoptosis, and senescence by interacting with various proteins, including p53, Rb, and DAXX. It has also been shown to regulate transforming growth factor beta (TGFβ) signaling and this could represent another avenue by which the variants in *PML* predispose to PDB, given the important role that TGFβ plays in regulating bone turnover.

4.11 7q33 Locus

The 7q33 locus was identified as a genetic determinant of susceptibility to PDB by an extended GWAS.[32] This locus contains three known genes (*CNOT4*, *NUP205*, and *SLC13A4*) and two predicted protein-coding transcripts (*PL-5283* and *FAM180A*). There is substantial linkage disequilibrium within this locus and variants in any of these genes could be responsible for the association observed. The strongest signal was within intron 22 of *NUP205*, which encodes the nucleoporin 205-kD protein. This is a component of the nuclear pore complex, which is involved in the regulation of transport of substances between the cytoplasm and nucleus. None of the genes located within this locus have so far been implicated in the regulation of bone metabolism and the mechanisms underlying the association remain to be established.

4.12 Other Susceptibility Genes and Loci

Several other candidate genes and loci have been implicated in the pathogenesis of PDB. The first candidate locus to be implicated in PDB was the human leukocyte antigen (HLA) region on chromosome 6,[70] but this finding has not been replicated by other linkage studies or GWAS, and it seems likely to be a false positive. Similarly, the 2q36 locus described by Hocking et al.[40] in patients of British descent with PDB seems to have been a false positive, as subsequent linkage studies in British families without *SQSTM1* mutations showed no evidence of 2q36 linkage.[37] A susceptibility locus for PDB (LOD score 3.7) was identified on chromosome 5q31 in the French–Canadian population.[39] Although the evidence for linkage was strong, the 5q31 locus did not emerge as a significant determinant of PDB in linkage studies performed in patients of British descent or in GWAS.

5 ANIMAL MODELS

Several animal models for PDB have been generated, some of which have explored the hypothesis that viral proteins cause PDB and others that have attempted to model human mutations associated with PDB.

5.1 Models of PDB Mimicking Measles Infection

One of the first animal models of PDB to be generated by overexpressing the measles virus nucleocapsid

protein (MVNP) in cells of the osteoclast lineage by using the tartrate-resistant acid phosphatase (TRAP) promoter.[71] Histological analysis of the vertebrae from MVNP-overexpressing mice showed evidence of increased bone turnover, which was reported to affect about 50% of the vertebrae analyzed. Osteoclast precursors from these mice have increased sensitivity to $1,25(OH)_2D_3$ and form hypernucleated osteoclasts as compared with osteoclasts from wild-type (WT) littermates. The osteoclasts produce increased amounts of interleukin-6 (IL-6) in vitro and osteoclast formation is inhibited by antibodies to IL-6, indicating that the enhanced osteoclast activity mediated by overexpression of the MVNP protein is due, in part, to overproduction of IL-6.

5.2 Genetic Models of PDB

5.2.1 *SQSTM1*

Several mouse models of *SQSTM1*-mediated PDB have been generated. The first attempted model of *SQSTM1*-mediated PDB was generated by using the TRAP promoter to overexpress the human P392L mutation in mice.[72] Osteoclast precursors from the P392L-overexpressing mice showed increased sensitivity to RANKL in vitro and histological analysis showed evidence of increased osteoclastic bone resorption, which was most marked in older animals. Bone volume was reduced in the P392L mutant animals of all ages when compared with WT, but there was no consistent effect on bone formation and no focal lesions were detected. This led the authors to suggest that the P392L mutation was not sufficient to induce a Pagetic phenotype in vivo. Subsequently, two groups of investigators generated a mouse model of PDB in which the proline residue at codon 394 of p62 was replaced by a leucine residue, resulting in a P394L mutation in the mouse germline equivalent to the P392L mutation in humans. Hiruma et al.[73] reported that osteoclast precursors from *sqstm1* P394L mutant mice were hypersensitive to RANKL and $1,25(OH)_2D_3$ and that stromal cells supported osteoclast formation more efficiently than WT littermates through increased RANKL production. Despite this, the mutant mice had no evidence of PDB-like bone lesions on histological examination of the lumbar spine. In contrast, Daroszewska et al.[74] generated a similar P394L model and found evidence of focal bone lesions characteristic of PDB mainly targeting the lower limbs. The lesions were characterized by increased osteoclastic bone resorption and increased bone formation with woven bone. The osteoclasts within lesions were hypernucleated and some contained nuclear inclusion bodies similar to those observed in humans with PDB (Fig. 25.3). The difference in skeletal phenotype between the models generated by these two groups of investigators may be attributed to the fact that in the Hiruma et al. study, analysis was limited to the vertebrae, whereas in Daroszewska et al. study, the lesions predominantly targeted the hind limbs.

5.2.2 *VCP*

Two animal models have also been developed for PDB associated with VCP mutations. A transgenic mouse model has been generated in which the R155H and A232E mutations were overexpressed, driven by the cytomegalovirus promoter.[75] These mice develop a myopathy with inclusion bodies and display behavioral abnormalities. Bone phenotyping was not performed. Another group of investigators generated the R155H model of IBMPDF using a knock-in strategy.[76] These mice also developed myopathy with inclusion bodies similar to those observed in the human disease. The muscle cells also showed increased rates of apoptosis and raised levels of the LC3B protein, consistent with an elevation in autophagic flux. Analysis of brain tissue also revealed the presence of inclusion bodies. Osteoclast precursors from mutant mice showed enhanced sensitivity to RANKL, and the mice were reported to have focal bone lesions characterized by increased osteoclast and osteoblast activities.

5.2.3 *OPTN*

The skeletal phenotype of an animal model homozygous for mutation in optineurin has been reported.[38] This mutation, an aspartic acid to asparagine change at codon 477 of the protein (p.D477N), causes loss of function for downstream signaling to TANK-binding kinase 1 (TBK1), as the mutated optineurin is unable to bind lysine 63–linked ubiquitin chains.[77] The D477N mutant mice had a generalized increase in bone turnover with increased osteoclastic bone resorption and bone formation.[38] The osteoclast precursors from these animals were hypersensitive to RANKL stimulation, but only 10% of mice aged greater than 15 months developed focal PDB-like lesion. These observations led the authors to conclude that while optineurin plays a key role in suppressing bone turnover, this particular loss-of-function mutation was not sufficient to cause a PDB-like phenotype.[38]

5.3 Complex Disease Models

In another model of PDB generated by Kurihara et al.,[78] the MNVP gene was overexpressed in mice with a germline P394L mutation of *sqstm1*. The mice showed focal osteolytic bone lesions in the spine in which bone turnover was markedly increased with production of woven bone. The osteoclast precursors from these animals were hypersensitive to the effects of RANKL and $1,25(OH)_2D_3$. In agreement with previous studies of the MVNP-overexpressing mice, IL-6 production was

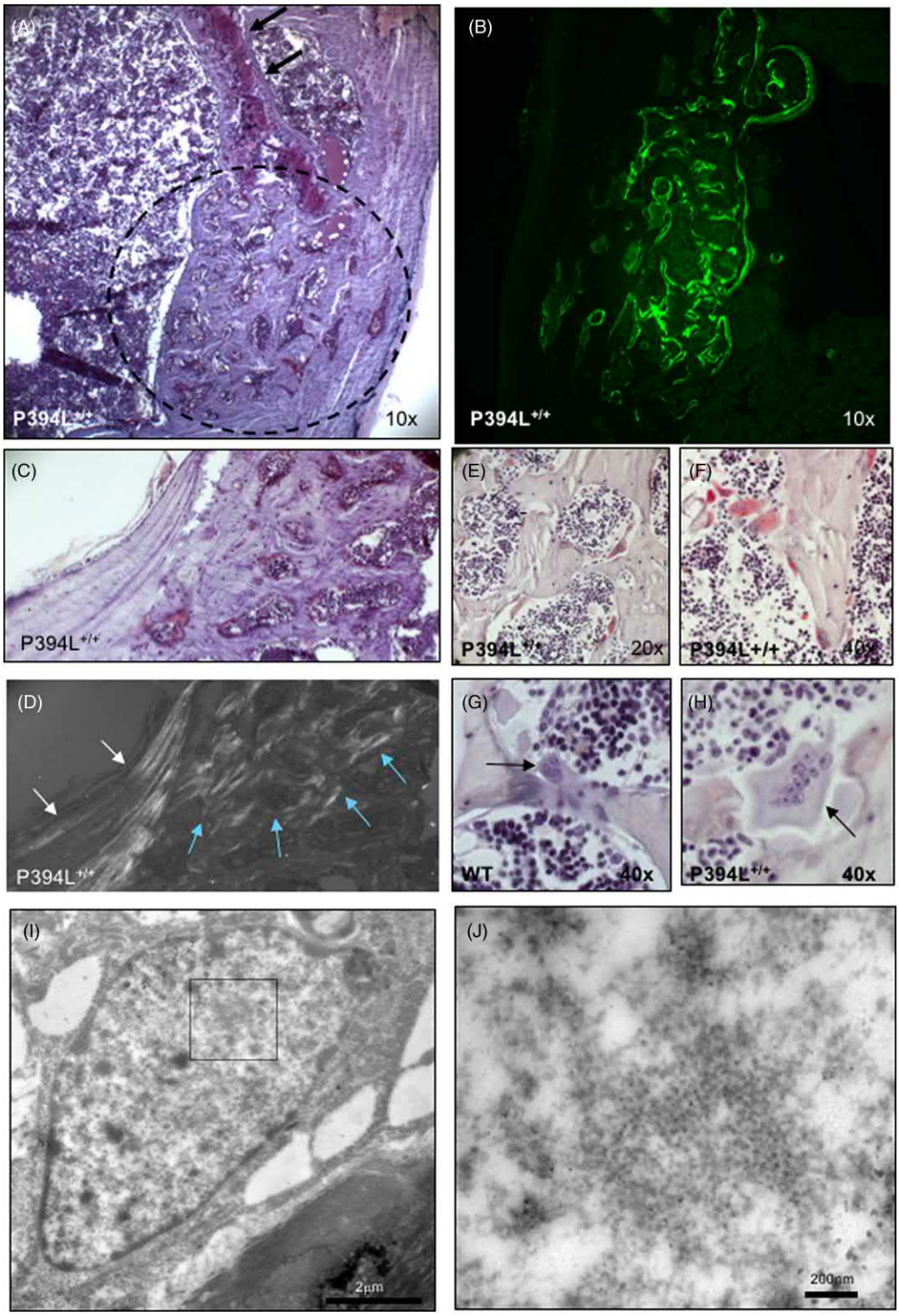

FIGURE 25.3 Bone lesions in P394L mutant mice. (A) Low-power photomicrograph of bone lesion *(circled)* stained for tartrate-resistant acid phosphatase (TRAP) and counterstained with hematoxylin showing a mixed osteolytic and osteosclerotic lesion in the trabecular compartment and cortex. (B) Photomicrograph of the same lesion visualized under fluorescent light, demonstrating markedly increased rates of bone formation, as reflected by the extensive calcein double labeling of bone surfaces *(green in the web version)*. (C) TRAP-stained section with a hematoxylin counterstain of a PDB-like lesion. Areas undergoing active resorption are stained (*red in the web version)*. (D) The same lesion seen under polarized light; the *arrows*on the left (*white in the web version)* point to lamellar bone, whereas the *arrows* on the right (*blue* in the web version) point to a large area of woven bone. (E) Photomicrograph of a lesion at 20× magnification, demonstrating increased bone resorption with multiple osteoclasts. (F) Higher-power view (40×) of a different area in the same lesion. (G) A normal osteoclast containing three visible nuclei from a wild-type (WT) animal. (H) A large osteoclast within a lesion, containing 10 visible nuclei, from a $P394^{+/+}$ mouse. (I) Transmission electron micrograph (TEM) image of osteoclast nucleus from a focal bone lesion in a $P394^{+/+}$ mouse, showing nuclear inclusions consisting of multiple microcylindrical structures *(dotted rectangle)*. (J) High-power TEM image of the nuclear inclusion shown in panel I. *Source: Reproduced from Daroszewska A, van't Hof RJ, Rojas JA, et al. A point mutation in the ubiquitin-associated domain of SQSMT1 is sufficient to cause a Paget's disease-like disorder in mice.* Hum Mol Genet *2011;***20***(14):2734–44, with permission.*[74]

markedly increased in bone marrow cultures from MVNP animals, both in the presence and absence of the P392L mutation. Kurihara et al. crossed the MVNP-overexpressing mice with IL-6 knockout mice and found that this abolished the hypersensitivity to $1,25(OH)_2D_3$ in vitro. This also is in keeping with previous studies in MVNP mice, which showed than IL-6 antibodies reduced osteoclast formation in marrow cells from mice that expressed the transgene.

6 MOLECULAR PATHOLOGY

6.1 Cellular Abnormalities

Paget's disease is generally considered to be a disorder of osteoclast differentiation and function.[79] The osteoclasts within Pagetic bone lesions increase in number and size and contain more nuclei than normal. Bone marrow cells derived from patients with PDB have increased sensitivity to $1,25(OH)_2D_3$ and receptor activator of RANKL as compared with normal bone marrow.[80,81] Nuclear inclusion bodies have also been identified in osteoclasts from PDB patients.[82,83] These were initially thought to represent paramyxovirus nucleocapsids,[82] but it has been more recently suggested that the inclusions may represent abnormal protein aggregates due to defects in the autophagy pathway.[84]

Abnormalities of bone marrow stromal cells and osteoblasts have also been described in PDB. Stromal cells from patients with PDB can better support osteoclast formation than those from controls,[85] and this is due in part to increased RANKL expression.[80] Patterns of gene expression have also been shown to differ in osteoblasts cultured from PDB patients and controls. In one study,[86] evidence for increased mRNA expression of IL-1, IL-6, Dickkopf1, and alkaline phosphatase in cultures from PDB patients was observed. This led the authors to speculate that osteoblast secretion of IL-1 and IL-6 might contribute to stimulation of osteoclast activity in PDB and that Dickkopf1 might be involved not only in promoting osteolysis but also in regulating osteoblast differentiation and mineralisation.[86]

6.2 Cytokines and Growth Factors

Some investigators have focused on the possibility that abnormalities of osteoclast formation in PDB may be driven in part by overproduction of cytokines or growth factors. One of the most widely studied cytokines is IL-6, which was reported to be in increased concentrations in plasma and conditioned media from Pagetic bone marrow cultures when compared with control cultures.[87] However, other investigators have found no difference in circulating IL-6 levels in Paget's patients when compared with controls[36] and no evidence of increased IL-6 mRNA expression in Pagetic bone.[88] Serum levels of fibroblast growth factor 2 (FGF2) have also been reported to be elevated in patients with PDB[89] and FGF2 has been found to increase RANKL expression in bone marrow cells. This led to the suggestion that FGF2 could predispose an individual to PDB by enhancing RANKL expression in the bone microenvironment; although the association between raised FGF2 levels and PDB remains to be confirmed in other studies. Raised circulating levels of the cytokine M-CSF were previously reported in a small cohort of untreated patients with PDB as compared with controls[36] and this observation has recently been confirmed in another study (Ralston & Albagha unpublished). These data are consistent with the hypothesis that PDB may be due in part to dysregulation of M-CSF production possibly mediated by genetic variations at the *CSF1* locus.[33]

6.3 Intracellular Signaling Pathways

Several genes associated with PDB directly affect the intracellular signaling pathways that regulate osteoclast activity. An important mechanism by which *SQSTM1* mutations cause osteoclast activation involves the deubiquinating enzyme, CYLD.[44,90] Under normal circumstances CYLD is recruited to the intracellular domain of the RANK receptor by p62 through its UBA domain. Deletion of the UBA domain of p62 and the P392L mutation impair the ability of p62 to recruit CYLD, which prevents deubiquitination of TRAF6 causing stimulation of NFκB signaling and osteoclast activation (Fig. 25.2). Studies have also been performed to investigate the mechanisms by which optineurin regulates osteoclast function. These have shown that optineurin normally inhibits NFκB signaling by a mechanism that is mediated in part through recruitment of CYLD and also by the upregulation of IFNβ production.[38]

Abnormalities in autophagy have also been implicated in the pathogenesis of PDB.[84] Many of the genes that have been implicated in PDB, including *SQSTM1*, *VCP*, and *OPTN*, are known to be involved in regulating ubiquitin-mediated protein degradation.[84,91] Mice with knockin of the P394L *Sqstm1* mutation have increased levels of mRNA for *sqstm1*, autophagy-related gene 5 (atg5), and light chain 3 gene (lc3) in osteoclast precursors and increased levels of LC3-II protein, consistent with enhanced autophagosome formation.[74] Furthermore levels of p62 protein have been reported to be elevated in peripheral blood cells and osteoclasts from patients with PDB, regardless of *SQSTM1* mutation status.[92] Although all of these observations indicate that autophagy is dysregulated in PDB, it is currently unclear to what extent this is a bystander phenomenon or whether it is causally related to the osteoclast activation.

6.4 Somatic Mutations

The focal nature of PDB has led to the suggestion that somatic mutations may also contribute to the disease and account for its unusual distribution.[93] However, studies that have sought to detect somatic mutations in PDB have yielded conflicting results. Following the identification of *TNFRSF11A* mutations in FEO and early-onset PDB, Sparks et al. probed for evidence of somatic mutations in affected tissue from PDB patients and in a variety of osteosarcoma cell lines, but none were detected.[94] In another study, evidence was presented to suggest that the P392L mutation of *SQSTM1* might be present in affected bone from some PDB patients and in some Pagetic osteosarcomas.[95] The signal for the mutant allele in this study was weak, indicating that if somatic mutations were present, they must have affected a very small number of cells. Another study using similar techniques found no evidence of somatic mutations in cultured bone marrow cells or cultured osteoblasts from affected bone in PDB.[96]

7 MOLECULAR DIAGNOSIS

Genetic testing for susceptibility to PDB and related disorders is frequently performed for the rare early-onset high-penetrance diseases, such as IBMPFD, JPD, and FEO. It has been suggested[97] that patients with classical PDB should also be offered genetic testing for *SQSTM1* mutations, as individuals that test positive are at a high risk of developing PDB and have more severe disease than patients negative for *SQSTM1* mutations.[98,99] At present, genetic testing for *SQSTM1* mutations in patients with PDB is not performed in routine clinical practice.

In the future it's possible that the risk of PDB could be assessed by genotyping for a panel of common alleles that predispose an individual to the disease. In case–control studies the common genetic variants identified by GWAS were associated with a modest increase in risk of PDB (1.4- to 1.7-fold), whereas the rare variants identified in the *SQSTM1* gene are associated with a very substantial increase in risk since these mutations are very seldom observed in the general population. Although these findings are promising, further research will be required to evaluate the predictive value of these variants. At the present time a clinical trial is in progress (the ZIPP study; ISRCTN11616770) to determine if genetic testing followed by targeted intervention can prevent the development of PDB-like lesions. The trial involves conducting genetic testing for *SQSTM1* mutations in adult children of patients with PDB and randomizing positive individuals to receive zoledronic acid or placebo. The results of this study should be available in 2020 and will provide new information on whether genetic testing followed by targeted intervention can be used to favorably alter the natural course of PDB.

8 CONCLUSIONS

Over the past 2 decades genetic studies have revolutionized the understanding of the pathophysiology of PDB, as well as identified new genes and signaling pathways that regulate osteoclast differentiation and function. Despite these advances, the environmental triggers for PDB are poorly understood and the mechanisms by which they interact with genetic variants to cause the disease are obscure. It is still unclear why PDB targets some bones and not others. Further research to clarify these issues will be of relevance not only in gaining greater understanding of PDB, but also in clarifying disease mechanisms in other conditions that target the skeleton in a focal manner.

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CHAPTER

26

Mendelian Disorders of RANKL/OPG/RANK/NF-κB Signaling

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1 INTRODUCTION

Identification of the RANKL/OPG/RANK/NF-κB signaling pathway as a major regulator of osteoclast (OC) formation and action and consequently skeletal remodeling began in 1997–98 with the discoveries of two additional receptors (RANK and OPG) and one additional ligand (RANKL) in the tumor necrosis factor (TNF) superfamily.[1] Soon after, studies of genetically altered mice revealed an unanticipated crucial role for these proteins in bone biology and helped demonstrate that binding of RANKL to RANK promotes osteoclastogenesis and OC action, and thereby increases skeletal turnover, whereas coupling of RANKL instead to its "decoy receptor" OPG attenuates this response.[2] Then, the importance of RANKL/OPG/RANK/NF-κB signaling for humans became strikingly illustrated by the discoveries of the genetic bases for several extremely rare heritable bone disorders.[3–8] These highly instructive and seemingly distinctive maladies of the skeleton, and sometimes also of teeth and vasculature, involve germline mutations that: (1) constitutively activate RANK[3,4,8] or cause OPG deficiency[5] thereby increasing osteoclastogenesis and accelerating skeletal turnover diffusely resembling what occurs focally in classic Paget's disease of bone (PDB),[9] or (2) deactivate RANKL[6] or RANK[7] thereby impairing osteoclastogenesis and causing osteopetrosis (OPT). Revelation of the etiology of these disorders began in 2000 when Hughes et al.[3] discovered that autosomal dominant (AD) familial expansile osteolysis (FEO) and an early-onset form of PDB in Japan (PDB2) were caused by heterozygous 18-bp and 27-bp tandem duplications, respectively, in the gene that encodes RANK (*TNFRSF11A*: OMIM 603499).[10] This advance provided important impetus to further explore the etiology of PDB (Chapter 25). Then, in 2002, Whyte and Hughes[4] reported that an AD entity they identified and called expansile skeletal hyperphosphatasia (ESH) in 2000[11] was caused by a 15-bp duplication in *TNFRSF11A*. Also in 2002, Whyte et al.[5] discovered that autosomal recessive (AR) selective deletion of the gene that encodes OPG (*TNFRSF11B*: OMIM 602643)[10] caused juvenile Paget's disease (JPD). Now, JPD worldwide is known to typically reflect homozygous loss-of-function *TNFRSF11B* founder mutations.[12] In 2014, Schafer et al.[8] found that a 12-bp duplication in *TNFRSF11A* caused an extraordinary disorder we called panostotic expansile bone disease (PEBD). Also in 2014, we identified a novel 15 bp duplication in *TNFRSF11A* in a Bolivian girl with what we called JPD2.[13] Each *TNFRSF11A* duplication occurred within exon 1. They would elongate the signal peptide of RANK by 6, 9, 5, or 4 amino acid residues, respectively.[3,4,8,13] Transfection of several of these mutated *TNFRSF11A*s showed the extended signal peptide resisted proteolytic cleavage, trapping the nascent RANK receptor intracellularly.[3] Seemingly, the shorter the duplication the earlier the disease presentation and more severe the skeletal findings. In stark contrast to these *TNFRSF11A* and *B* mutations that promote osteoclastogenesis and OC action, Sobacchi et al.[6] and Guerrini et al.[7], respectively, discovered AR loss-of-function mutations in RANKL (*TNFSF11*: OMIM 602642)[10] and RANK (*TNFRSF11A*) that impair osteoclastogenesis and cause "OC-poor" forms of OPT. In 2016, mutation within the five-member NF-κB complex itself was implicated in skeletal disease when Frederiksen et al.[14] reported a neonate with high bone mass and a heterozygous loss-of-function mutation of *p65* encoding RELA. Likely, additional new entities will be recognized.

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Herein, I provide an overview of the Mendelian disorders that illustrate the importance of RANKL/OPG/RANK/NF-κB signaling. They are discussed according to the specific gene involved: *TNFRSF11A* (RANK) for FEO, PDB2, ESH, PEBD, and JPD2; *TNFRSF11B* (OPG) for JPD1; *TNFSF11* and *TNFRSF11A* for the OC-poor OPTs; and *p65* (RELA) for neonatal high bone mass.

2 DISORDERS FROM CONSTITUTIVE RANK ACTIVATION

2.1 Familial Expansile Osteolysis

FEO is sometimes called hereditary expansile polyostotic osteolytic dysplasia (OMIM #174810).[10] Severely affected individuals manifest early-onset deafness from destruction of inner ear bones, and then resorption of adult (secondary) dentition together with focal progressive osteolytic expansion of major bones in the limbs leading to pain, fractures, and deformities.[15,16] Osteosarcoma may develop.[17,18] Early on, an osteolytic FEO lesion has clinical, radiographic, and histopathological similarities to the active (osteolytic) phase of classic PDB (OMIM #167250),[9,16] but progresses instead to expanded shell-like bone that is fat-filled rather than the disorganized and sclerotic "mosaic bone" characteristic of PDB.[9]

FEO was first mentioned in 1976 in a brief communication from Northern Ireland by Osterberg ($FEO_{(NI)}$).[19] However, characterization of FEO itself began instead with dual publications by Enderle and coworkers in 1979 concerning two brothers in Germany ($FEO_{(Ger)}$) who were unrelated to $FEO_{(NI)}$ and suffered an unusual "osteolytic-expansive" PDB later recognized as FEO (see later).[17,18] Characterization of $FEO_{(NI)}$ actually began with the 1987 medical thesis of Wallace[20] and became fully detailed by 7 subsequent reports that described 46 affected family members in the 5-generations $FEO_{(NI)}$ kindred.[21–27] In 1994, $FEO_{(NI)}$ was mapped by Hughes et al.[28] to chromosome 18q21.1-q22. A clinical review of FEO was published in 1996 by Hughes and Barr.[15]

Then, in 2000, the genetic basis for $FEO_{(NI)}$ was identified using a candidate gene approach.[3] At that time, RANKL (then also called "osteoclast differentiation factor" or "OPG ligand") was understood to be a paracrine factor of marrow mesenchyme-derived cells and activated T-cells.[1,2,29] Hughes et al. found[3] in $FEO_{(NI)}$, $FEO_{(Ger)}$, and our yet to be reported[16] American kindred [$FEO_{(Am)}$] an identical, in-frame, 18-bp, tandem duplication (84dup18) in exon 1 of *TNFRSF11A* that encodes the signal peptide of RANK. Their transfection studies indicated that its predicted hexapeptide extension rendered FEO-RANK resistant to cleavage and trapped it intracellularly somehow causing gain-of-function and enhancing NF-κB activity.[3] Then, in 2002, Whyte and coworkers[16] reported their 30-year experience with the 5-generation $FEO_{(Am)}$ kindred and provided a clinical overview of FEO. In the 3 FEO kindreds there were 3, 46, and 8 affected individuals, respectively,[16–18,21–27] who were unrelated according to genealogy and haplotype analysis. Subsequently, in 2002, a Spanish kindred with mild FEO[30] [$FEO_{(Sp)}$] was reported with 20 affected individuals. In 2003, two unrelated American FEO patients were described.[31] In 2007, Elahi et al.[32] reported that intragenic SNP haplotypes found with the 84dup18 mutation in *TNFRSF11A* in the four FEO kindreds suggested three independent origins for this mutation. The principal features of the four FEO kindreds are reviewed, contrasted, and further discussed below.

2.1.1 $FEO_{(Ger)}$

The two brothers with $FEO_{(Ger)}$ reported by Enderle and coworkers in 1979[17,18] had similar findings considered an "osteolytic-expansive" familial PDB in the "initial-active" phase that failed to progress to the "inactive" phase. The propositus had expanded fibulas, radii, and patellas at approximately age 25 years. Their father, who died at age 47 years, was probably affected because he succumbed to metastatic osteosarcoma including focal skull lesions (see later). However, there was no mention of deafness, dental problems, or generalized osteopenia in $FEO_{(Ger)}$. Light-microscopy of the skeleton showed filigree-like trabeculae comprised of woven bone without a mosaic pattern, an abundance of giant OCs with bizarre shapes and numerous nuclei, significant subperiosteal bone formation, and fibrous marrow. $FEO_{(Ger)}$ seems to represent the most severe FEO by featuring unusually symmetrical polyostotic disease in the extremities involving especially the radii and fibulas.[17,18] However, too little was reported about $FEO_{(Ger)}$ to know if other principal clinical features of FEO (see later) were present.

2.1.2 $FEO_{(NI)}$

The eight publications that characterized $FEO_{(NI)}$ commenced in 1987[20] with Wallace's medical thesis that emphasized the clinical and radiographic features, and later comprised seven studies by several medical subspecialties.[21–27] Nearly complete penetrance of $FEO_{(NI)}$ was documented. However, severity differed among the family members. With few exceptions affected individuals suffered three principal complications. First, conductive deafness that became mixed-type troubled at least 95% of these subjects.[26,27] Deafness could present as early as 4 years-of-age,[26] but more commonly was detected during the second decade-of-life.[23] The long process of the incus was usually absent or replaced by fibrous tissue.[22,26,27] Second, 90% of these individuals had progressive osteolytic lesions that were especially common in the lower limbs and typically multifocal.[22,26] A tibia was always involved.[26,27] Most of these lesions resembled classic PDB

by beginning at (or near) the end of a long bone,[27] then advancing relentlessly[22] 6.5–22 mm yearly.[23,26] The maximum number of lesions in one person was 12.[27] Skeletal pain began between ages 18 and 44 years, sometimes leading to limb amputation.[22,23,25,26] Third, most affected individuals had loosening and pain and/or fracture of secondary (adult) teeth,[26,27] however, the primary (deciduous) teeth were not involved.[25] Several family members with $FEO_{(NI)}$ also suffered malignant transformation of bone.[22]

Radiographic abnormalities in $FEO_{(NI)}$ included modeling distortions of some bones, especially the humerus, radius, ulna, and tibia. Also, a tightly meshed ('fish-net) trabecular pattern was variably present throughout the skeleton,[22,23,26] particularly in metaphyses,[26] that sometimes was also observed in the mandible.[23] However, generalized osteopenia was not reported. Bone scintigraphy showed proportionately greater radio-isotope uptake in the tibias compared to the femurs.[26] Bone turnover markers (BTMs) indicated accelerated skeletal remodeling.[26]

The histopathology of the early osteolytic lesions in $FEO_{(NI)}$ resembled PDB.[21,26,27] The OCs were particularly large with increased numbers of nuclei.[27] All of the OCs studied by electron microscopy had microcylindrical nuclear inclusion bodies, that resembled measles, canine distemper, or respiratory syncytial virus.[21,23,27] However, intermediate-stage disease instead showed scanty skeletal matrix with increased fibrous tissue and extensive vascularity. Fat occupied the medullary spaces.[22,26,27] Advanced lesions featured nearly complete loss of cortical and trabecular bone. In contrast, light microscopy of the iliac crest was unremarkable.[27]

Investigation of the dentition of 27 individuals with $FEO_{(NI)}$[24] revealed histological disturbances that featured reduction in the size of pulp chambers and root canals, patchy narrowing of the periodontal ligament, and extensive root resorption.[25] Nevertheless, the most remarkable and unique complication was designated "idiopathic external resorption" (IER)[25] that occurred at the enamel/cementum junction and eventually decapitated the tooth (see later).

The brief earliest 1976 report of $FEO_{(NI)}$ mentioned symptomatic relief (without radiographic change) during synthetic salmon calcitonin (CTN) therapy.[19] Later, dichloromethylene diphosphonate was not effective.[27] Intravenous infusions of pamidronate (PMD) seemed most helpful.[22,27] However, antiresorptive therapy was considered unsatisfactory overall.[22]

2.1.3 $FEO_{(Am)}$

In 2002,[16] our 30-year experience with $FEO_{(Am)}$ confirmed that excessive RANK activity could cause early-onset deafness, loss of adult dentition, and osteolytic expansion of major appendicular long bones (Fig. 26.1). Additionally, generalized osteopenia was recognized as a common and sometimes important fourth feature of FEO in adults.[16] The accelerated bone remodeling of FEO caused a type of "high-turnover" osteoporosis, sometimes with fractures. However, investigation of $FEO_{(Am)}$ revealed that this FEO was significantly milder than $FEO_{(Ger)}$ or $FEO_{(NI)}$, and that alendronate (ALD) therapy could suppress to normal the elevated BTMs in affected adults and heal early osteolytic lesions (see later).[16] In fact in $FEO_{(Am)}$, the four major complications of FEO manifested in only some individuals, and from further experience (unpublished) with the kindred, there are asymptomatic elderly carriers of the 18 bp RANK mutation. Deafness was the first symptom, generally presenting from early childhood to young adult life, but perhaps not until age 60 years.[12] The propositus' medical record did not mention deafness, although hearing loss due to degeneration of middle ear bones troubled nearly all affected family members.[28] However, premature tooth loss was exceptional in $FEO_{(Am)}$.[12] At age 29 years, one affected man denied skeletal symptoms and had suffered only tooth loss from IER (Fig. 26.2). An obligate carrier woman, who lived 89 years, reportedly had deafness and tooth loss late in life, yet no symptoms of osteolytic disease. Of interest, experience with $FEO_{(Am)}$ suggested that skeletal trauma (including orthodonture), perhaps by activating OCs, could incite lytic lesions (see later).[16]

In $FEO_{(Am)}$, serum alkaline phosphatase (ALP) became elevated during adolescence and represented a

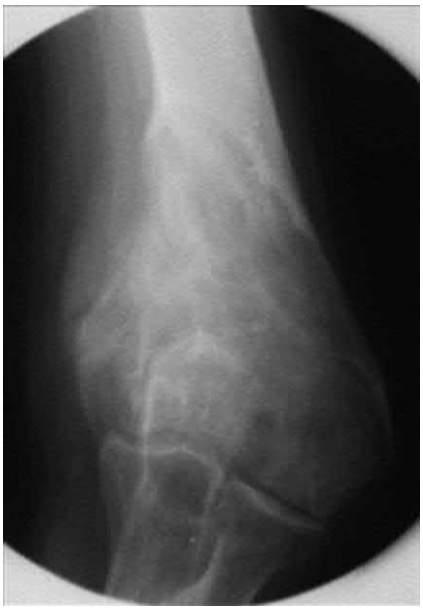

FIGURE 26.1 Osteolytic/expansile lesion of familial expansile osteolysis (FEO). The 71-year-old propositus with $FEO_{(Am)}$[16] presented with this osteolytic/expansile (soap bubble) lesion in his left distal humerus. The radius and ulna are osteopenic with coarse trabeculae likely reflecting disuse atrophy. *Source: Reproduced from Whyte MP, Mumm S. Heritable disorders of the RANKL/OPG/RANK signaling pathway.* J Musculoskelet Neuronal Interact *2004;***4***:254–67, with permission.*

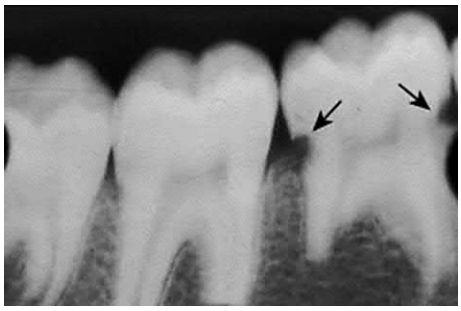

FIGURE 26.2 Tooth destruction in FEO. This dental radiograph of a 14-year-old boy with $FEO_{(Am)}$[16] shows characteristic "idiopathic external resorption" (IER) of a tooth (*black arrows*) where the root is also being resorbed. *Source: Reproduced from Whyte MP, Mumm S. Heritable disorders of the RANKL/OPG/RANK signaling pathway.* J Musculoskelet Neuronal Interact *2004;**4**:254–67, with permission.*

reliable BTM signifying rapid skeletal remodeling.[16] Hyperphosphat*asemia* typically preceded radiographic skeletal changes. The bone disease seemed to first destroy middle ear ossicles, causing deafness by early childhood. Accelerated turnover then led to generalized osteopenia, and probably contributed to the characteristic coarse trabecular pattern seen radiographically as early as age 20 years. Osteolysis elsewhere could begin during young adult life or middle age, and before hyperphosphatasemia, osteopenia, or a coarse trabecular pattern were present.[16] Osteopenia distal to expansile lesions was common, and likely in part represented disuse atrophy of bone. Four individuals with $FEO_{(Am)}$, ages 27–67 years, underwent biopsy of an expansile lytic lesion showing penetration by fibrous structures that often involved most of the bone. Light microscopy of the osteolytic defects was always typical of early classic PDB (Fig. 26.3). OC-mediated bone resorption, marrow fibrosis, and membranous bone formation were present. Eroded areas of fibrous trabeculae had foci of numerous enlarged giant cells with many nuclei, some nestled in Howship's lacunae, that appeared like the OCs reported in PDB. However, these bony defects could be unlike PDB because they also lacked "cutting cones" containing concentrations of OCs, and had scattered OCs. Importantly, "mosaic bone" was rarely seen. Instead, woven bone appeared to be rapidly deposited and, even in older areas, failed to extensively remodel into mature cortical bone to produce skeletal "healing" with osteosclerosis.[16] Electron microscopy revealed only rare inclusion bodies (see later).[16] Generally, unless antiresorptives had been administered, there was little radiographic osteosclerosis that characterizes advanced PDB. Iliac crest specimens from three affected individuals showed no features of PDB.[16]

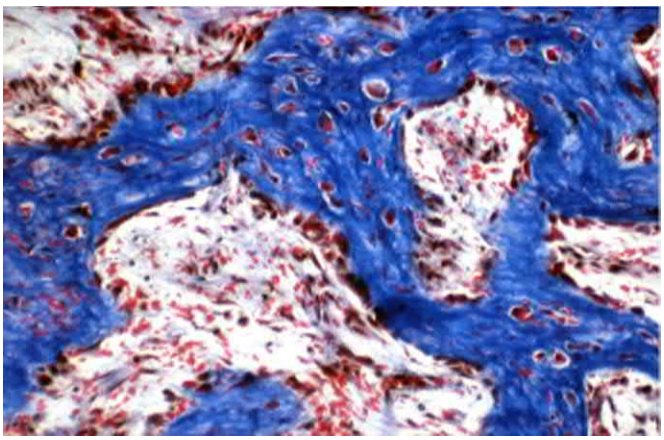

FIGURE 26.3 Bone histology of osteolytic/expansile lesion of FEO. This undecalcified section of an osteolytic lesion of the distal humerus of a woman with $FEO_{(Am)}$[16] shows woven bone, fibrous tissue in the marrow spaces, and numerous osteoclasts (OC) (Masson trichrome stain, ×80). *Source: Reproduced from Whyte MP, Mumm S. Heritable disorders of the RANKL/OPG/RANK signaling pathway.* J Musculoskelet Neuronal Interact *2004;**4**:254–67, with permission.*

2.1.4 $FEO_{(Sp)}$

In 2002, Palenzuela et al.[30] reported a four-generation Spanish kindred with FEO [$FEO_{(Sp)}$] affecting 20 members who harbored the same 18-bp duplication (84dup18) in exon 1 of *TNFRSF11A* discovered in the first three FEO kindreds.[3] Hence, it was proposed that there is a "hot spot" for a meiosis-stable mutational event in exon 1 in the RANK gene,[30] but FEO is in fact remarkably rare.

Typically, the first symptom for $FEO_{(Sp)}$ was deafness presenting between ages 7–12 years. In the second decade-of-life, loss of teeth occurred, and then skeletal changes could appear. All affected individuals had hearing and dental abnormalities, elevated BTMs, radiologically apparent osteoporosis in the advanced years, but osteolytic/expansile bone lesions were uncommon (i.e., two individuals), with histopathology of one lesion showing a marked increase in bone remodeling with hypermultinucleated OCs, and pathological fractures did not occur.[30]

2.1.5 Additional Reports of FEO

In 2003, Johnson-Pais et al.[31] reported two unrelated Americans with FEO. Both had early hearing loss, deterioration of teeth, and osteolytic/expansile bone disease, but their *TNFRSF11A* mutation 83dup18 began one base pair proximal to the classic 84dup18 FEO mutation. Nevertheless, both duplications were predicted to insert the identical hexapeptide extension within the signal peptide of RANK.[3] These authors discussed a potential mechanism for mutation of exon 1 within *TNFRSF11A*.[31] Then in 2006, Marik et al.[33] reported a Czech girl with FEO but subsequently we did not find a mutation in exon 1 of *TNFRSF11A* encoding RANK or in *TNFRSF11B* encoding OPG (Whyte et al., unpublished).

In 2010, Bacri et al.[34] reported a chondroblastic osteosarcoma in a consanguineous adolescent boy in a Druze kindred with "FEO," but we subsequently found he has

JPD (see later) due to a homozygous unique *TNFRSF11B* mutation encoding OPG (Whyte et al., unpublished).

2.1.6 FEO Treatment

In 1988, medical treatment was considered unsatisfactory for $FEO_{(NI)}$, with intravenous infusions of PMD seemingly the best option.[26] The $FEO_{(Am)}$ patients had received antiresorptive pharmaceuticals as they became available.[16] Generally, CTNs and bisphosphonates (BPs) slowed skeletal turnover with different efficacy judged by reductions in BTMs. In some $FEO_{(Am)}$ patients, radiographic improvements of focal lesions were documented by partial healing of osteolytic fronts and their remineralization and cortical thickening.[16] Parenteral injections of synthetic salmon CTN for 1 year proved minimally helpful for one patient, with transient halving of serum ALP activity, but no beneficial clinical response. Nasal administration of synthetic salmon CTN seemed ineffective.[16] For several $FEO_{(Am)}$ patients, injections of synthetic human CTN were superior to salmon CTN in lowering serum ALP activity, correcting urinary hydroxyproline (OHP) levels, and producing cortical and trabecular thickening documented radiographically within lytic lesions.[16] However, these improvements were transient, with eventual resistance. Etidronate (EHDP) lowered serum ALP activity and urinary OHP levels and diminished radionuclide uptake on bone scintigraphy in one individual with $FEO_{(Am)}$. In two subjects with $FEO_{(Am)}$ and active osteolysis, BTMs normalized and small osteolytic defects healed following a 6-month course of ALD.[16] In an older patient, ALD corrected serum ALP activity as well as urinary OHP and deoxypyridinoline levels, but had no effect radiographically on a markedly expanded entire tibia with an "egg shell" appearance.[16] Perhaps such refractoriness to antiresorptive therapy for osteolytic lesions in FEO occurs when their osteoprogenitor cells no longer function and bones become fat-filled and "burned-out."[22,26,27] Nevertheless, bone density could improve elsewhere (e.g., lumbar spine).[16] Two years after withdrawal of ALD therapy, clinical, biochemical, and radiographic improvements with better BMD remained in one affected individual. A small lytic lesion within a distal humerus remineralized during a prolonged course of ALD. It seemed that ALD therapy during the early stages of $FEO_{(Am)}$, when osteolytic lesions most closely resemble active PDB, produced durable benefits. Furthermore, IER of teeth could be halted (unpublished). However, no $FEO_{(Am)}$ patient reported a change in their deafness.[16] Recently, I observed prolonged normalization of serum ALP levels after one zoledronic acid infusion given despite advanced expansile disease (unpublished).

The antiresorptive monoclonal antibody against RANKL, denosumab, has not been tested in FEO, PDB2, or ESH (see later). In fact, the mutated RANK is trapped intracellularly and constitutively active.[3,35,36] However, individuals with FEO, PDB2, ESH, and PEBD carry one intact *TNFRSF11A* allele (see later) and its healthy RANK should reach cell surfaces and be therapeutically suppressed using denosumab that deactivates extracellular RANKL. However, hypocalcemia and rebound rapid bone remodeling would require careful consideration when starting and stopping treatment, respectively.

2.2 Early-Onset Paget's Disease of Bone (PDB2)

The genetic basis of PDB2 (OMIM: 602080)[10] was discovered in 2000 simultaneously when FEO became understood at the gene level.[3] In PDB2 the original Japanese family [$PDB2_{(Jpn)}$] with six affected members carried a 27 bp duplication in RANK (75dup27). The clinical features were detailed later in 2003 by Nakatsuka et al.[37] In 2009, a second PDB2 family was described by Ke et al.[38] but in China [$PDB2_{(Chn)}$] and involving a different 27 bp duplication (78dup27).

2.2.1 $PDB2_{(Jpn)}$

Affected family members had hearing loss presenting at 7–24 years-of-age.[37] Tooth loss began in the second or third decade-of-life. Bowing of the lower limbs also occurred at that time. Radiographs generally showed features of widespread PDB where large and small tubular bones were enlarged, deformed, and had "osteolytic" and osteosclerotic areas. Osteosclerosis of the mandible and maxilla was a distinctive feature in all affected individuals, and most also had asymptomatic bone enlargement at the small joints of the hands. Skull vault involvement was another typical finding.[37] Serum ALP activity was markedly elevated. On histopathology assessments, iliac crest bone was said to appear normal, or to show increased turnover, prominent OC activity, and woven bone.[37] The authors concluded that the unique duplication in *TNFRSF11A* (75dup27) caused a distinct PDB-like phenotype that overlapped with PDB, FEO, and ESH (see later). However, an important distinction was the later age-of-onset. FEO and ESH typically manifested with severe deafness before age 10 years, whereas the hearing impairment of $PDB2_{(Jpn)}$, although invariably present, was relatively mild and noted in the late teens or early 20s.[37] Also, in FEO and ESH early loss of dentition occurred during childhood or adolescence whereas in $PDB2_{(Jpn)}$ this presented in the late teens to mid-20s.[37] In $PDB2_{(Jpn)}$, mild expansion of the metacarpals and phalanges appeared on radiographs but, unlike in ESH,[11] this caused no symptoms. As in ESH, the $PDB2_{(Jpn)}$ propositus experienced at age 16 years transient hypercalcemia when immobilized from a sports injury.[37] However, this occurred uniquely during immobilization (a risk factor for hypercalcemia in both PDB and healthy adolescents). In 2008, Riches et al.[39] reported that $PDB2_{(Jpn)}$ responded with incomplete and short-lived reductions in BTMs

with high-dose EHDP therapy whereas amino-BP treatment led to greater and longer BTM suppressions. Nevertheless, no clear benefit followed BP therapy concerning bone deformity, deafness, or tooth loss, although bone pain improved in one of three affected individuals.[39] In 2007, a mouse model was developed for PDB2.[40]

2.2.2 *PDB2$_{(Chn)}$*

In 2009, Ke et al.[38] reported a Chinese family with PDB2 [PDB2$_{(Chn)}$] caused by a heterozygous 78dup27 duplication in *TNFRSF11A*, rather than the original 75dup27 duplication of PDB2$_{(Jpn)}$.[3] Both duplications would introduce a nonapeptide, albeit different ones, into the signal sequence of RANK. The PDB2$_{(Chn)}$ duplication was identified in four symptomatic individuals, and in one asymptomatic family member with elevated BTMs.[38] Striking involvement of the maxilla and mandible, skeletal pain, bowed legs, low bone density, fractures, and elevated BTMs were common features of FEO$_{(Chn)}$. Fingers were enlarged, but without pain. There was no deafness or tooth loss in childhood. The PDB2$_{(Chn)}$ phenotype was said to overlap both PDB and PDB2$_{(Jpn)}$, with the key difference compared to PDB2$_{(Jpn)}$[37] being the delayed onset, typically the late 20s, for PDB2$_{(Chn)}$.[38] PDB2$_{(Chn)}$ responded well to ibandronate administered intravenously.[38]

2.3 Expansile Skeletal Hyperphosphatasia

ESH was characterized in our publication from 2000 concerning the affected Australian mother and daughter (Fig. 26.4).[11] In 1991, Chosich et al.[41] had reported the mother as a case of postpartum hypercalcemia in JPD.

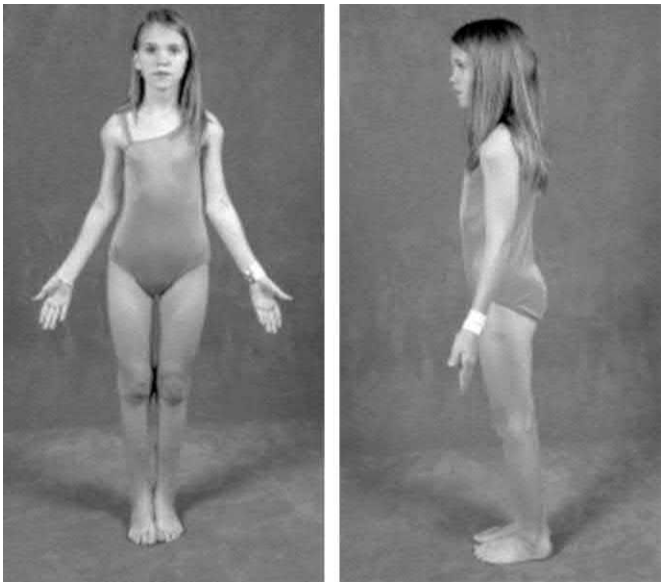

FIGURE 26.4 Physical findings of ESH during childhood. The 11-year-old daughter with ESH[11] has a broad forehead and some hypertelorism. She was deaf since infancy.

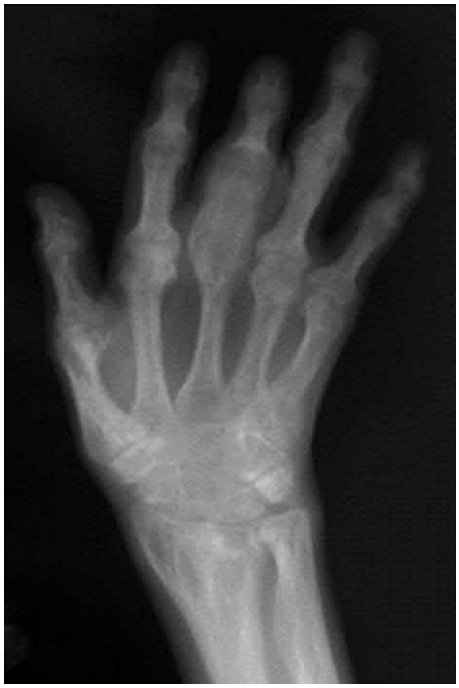

FIGURE 26.5 Expansile lesions of tubular bones in expansile skeletal hyperphosphatasia (ESH). This radiograph of a hand of the 36-year-old mother with ESH[11] shows a remarkably expanded proximal phalanx of the middle finger as well as areas of hyperostosis and irregular osteosclerosis. *Source: Reproduced from Whyte MP, Mumm S. Heritable disorders of the RANKL/OPG/RANK signaling pathway.* J Musculoskelet Neuronal Interact *2004;***4**:*254–67, with permission.*

Then, in 2002, Whyte and Hughes[4] discovered that ESH was an AD disorder caused by a heterozygous, 15-bp, tandem duplication (84dup15) in *TNFRSF11A*. Hence, FEO, PDB2, and ESH became allelic, indeed "exonic," diseases of RANK activation.[4]

ESH features deafness in infancy, premature loss of teeth, accelerated bone remodeling, with progressive hyperostotic widening of long major bones, painful expanded phalanges in the hands (Fig. 26.5), and episodic hypercalcemia.[11] The dental manifestations were detailed in 1999 by Olsen et al.[42] Episodic hypercalcemia together with absence of large osteolytic/expansile lesions in major long bones clinically distinguished ESH from FEO.[11] As in FEO,[16] deafness was the earliest problem in ESH.[11] Both mother and daughter developed hearing deficits in infancy, that were complete by early childhood. Radiographs of the daughter's skull were unremarkable at age 11 years,[11] and therefore compression of the VIIIth cranial nerve seemed excluded. Instead, the mother reportedly had no middle ear bones.[11] Necrotic degeneration of these structures is found in FEO,[43] and hearing loss is common from such conduction deficits also in JPD (see later).[5]

Further symptoms of ESH presented in childhood.[11] The mother developed symptomatic hypercalcemia at age 11 years.[41] Her daughter developed skeletal pain at

age 10 years soon after she fractured a wrist, perhaps provoking a generalized skeletal reaction. This included bony expansions of the proximal interphalangeal joints in the hands worrisome for arthritis. However, these small joints were not "hot" but could be tender. It was not known if any skeletal disturbance had been present earlier.[11,41] In contrast with PDB2,[37–39] the hands in ESH caused the greatest discomfort and deformity.[11]

Tooth loss during childhood or early adult life also characterized ESH.[42] The dental problems were detailed in 1999 and remarkably similar to FEO.[42] We did not find radiographic abnormalities in the mandible of the daughter at age 11 years,[11] although her mother was edentulous. Soon after, however, the daughter lost teeth.[42] In 1999, Olsen et al.[42] reported that both individuals manifested progressive root resorption of their adult teeth. The mother showed this problem at age 18 years, with all teeth extracted at age 28 years.[42] The daughter showed cervical root resorption in at least 13 teeth at age 12 years. The dental histopathology findings were initially considered consistent with JPD, but review of the mother's dental radiographs demonstrated findings in keeping with FEO.[42] Thus, secondary tooth loss characterizes FEO, PDB2, and ESH.

The mother's osteopathy was reportedly affected by intercurrent illnesses[11] and was shown to be influenced by pregnancy.[41] Her episodic hypercalcemia occurred spontaneously but also during illnesses in childhood and later during lactation. Chosich et al.[41] postulated in 1991 that the hypercalcemia reflected enhanced OC activity and uncoupling of bone remodeling, including from lowered estrogen levels during lactation, superimposed on the rapid skeletal turnover of JPD.[41] Serum ALP activity and additional BTMs were substantially elevated before antiresorptive therapy.[11] Episodic hypercalcemia also developed in her daughter.[42] ESH seemed a progressive disorder into early adult life.

Radiographic survey of the mother showed that ESH eventually disturbed the entire skeleton.[11] Major abnormalities featured expansion as well as hyperostosis (cortical thickening) of large and small tubular bones. Bowing of long bones, particularly the femurs, was present. Her calvarium (excluding the basal occiput), thoracic vertebral bodies, and long bones were affected most. However, the most striking change was phalangeal widening in her hands (Fig. 26.5). Findings in the daughter were similar, though considerably less severe.[11]

Iliac crest histopathology showed increased numbers of osteoblasts (OBs) and OCs.[11] However, the daughter's dynamic histomorphometry indicated a normal rate of bone accretion and total bone volume. In contrast, her mother who had received BP treatment years earlier showed total bone volume that was markedly increased,[11] bone resorption occurring primarily in cortical bone, no peritrabecular fibrosis, and reversal lines but not those of PDB.[9] There was erosion of cortical Haversian canals without corresponding bone loss in the trabecular compartment.[11] The histopathological changes were concordant with the radiographic findings. Possibly, her coarse linear trabeculae appeared enhanced by cortical bone loss providing a "window" on the trabecular struts.[11] Electron microscopy showed disorganized collagen bundles as well as necrotic and apoptotic bone cells.[11] Measles virus gene transcripts consistent with PDB were not detected in her peripheral blood monocytes.[11]

Although the mother's symptoms did not improve with salmon CTN, EHDP, or PMD therapy, her hypercalcemia seemed to respond to antiresorptive treatment.[11] Our suggestion to give ALD, based on favorable observations in $FEO_{(Am)}$,[16] led to symptom relief and normalization of her BTMs.[42]

Thus, ESH seemed distinctive from FEO partly because osteolytic expansion of major long bones is the hallmark of FEO,[16] but not a feature of ESH[11] where instead bones become broad and hyperostotic (Fig. 26.6). Furthermore, episodic hypercalcemia characterized ESH.[41] Nevertheless, ESH had sufficient likeness to FEO and PDB2 to suspect a similar etiology and pathogenesis. In 2002, we found a heterozygous, 15-bp, tandem duplication (84dup15) in *TNFRSF11A*[4] indicating that like FEO and PDB2,[3] ESH involved excessive RANK activity. Despite the phenotypic differences among FEO, PDB2, and ESH, the heterozygous mutations were remarkably similar overlapping 18-bp, 27-bp, and 15-bp tandem, in frame, duplications in exon 1 of *TNFRSF11A* (84dup18 or 83dup18, 75dup27 or 78dup27, and 84dup15, respectively). The duplications would lengthen the signal peptide of RANK by 6, 9, and 5 amino acids, respectively[3,4] and

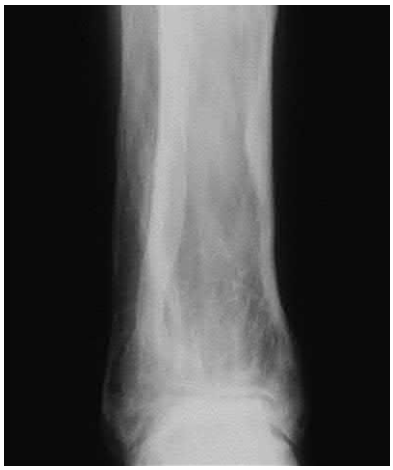

FIGURE 26.6 Expanded and hyperostotic long bone in ESH. This radiograph of a distal tibia of the 36-year-old mother with ESH[11] shows expansion (undertubulated) with irregular cortical thickening and a disorganized (coarse and radiolucent) trabecular pattern. Similar changes are seen in the fibula. *Source: Reproduced from Whyte MP, Mumm S. Heritable disorders of the RANKL/OPG/RANK signaling pathway.* J Musculoskelet Neuronal Interact *2004;***4***:254–67, with permission.*

interfere with the proteolytic cleavage of the RANK signal peptide and thereby trap the receptor intracellularly.[3,35,36]

2.4 Panostotic Expansile Bone Disease

In support of distinctive diseases arising from different length RANK signal peptide extensions, we detailed in 2014 the extraordinary, sporadic, PEBD with childhood deafness and with a massive jaw tumor in a young Mexican man.[8] We reported that he carried a heterozygous, 12-bp, tandem duplication (90dup12) in exon 1 of *TNFRSF11A*.[8]

2.5 Juvenile Paget's Disease, Type 2 (JPD2)

Finally, in 2014, we reported the JPD phenotype (see later) in a Bolivian girl with a unique, heterozygous, *TNFRSF11A* duplication (87dup15) predicting the same RANK signal sequence pentapeptide elongation found in ESH (see later).[13] In fact, her disorder seemed most like ESH, but she had received repeated courses of PMD that would obscure the natural phenotype. We called her disorder JPD2.

2.6 Other Phenotypes

However, with so few patients described worldwide that harbor these gain-of-function *TNFRSF11A* mutations, future reports will likely document further phenotypic overlap among the associated disorders, although new entities also seem possible from novel duplications.

Currently, we are studying additional candidate patients. We have found a novel 27 bp mutation in *TNFRSF11A* associated with a high-turnover but not yet fully characterized bone disease.[44] We did not find a *TNFRSF11B* mutation in the consanguineous patient with a polyosteolysis/hyperostosis syndrome reported by Kantaputra et al.[45] in 2006.

2.7 Pathogenesis From Constitutive RANK Activation

In 2000, Hughes et al.[3] reported transfection studies that showed increased NF-κB signaling by the FEO-RANK and PDB2-RANK mutations. The RANK signal peptide extensions seemed to prevent proteolytic cleavage, thereby trapping the nascent RANK intracellularly and somehow enhancing NF-κB signaling. In contrast, in a preliminary report from 2007, mice that overexpressed homozygous PDB2 mutations abrogated NF-κB signaling and suffered lethal OPT.[40] In 2011, Crockett et al.[35,36] confirmed that nascent FEO-RANK, PDB2-RANK, and ESH-RANK sequester intracellularly and therefore would not bind extracellular RANKL. When overexpressed, these three RANK molecules remained in the "organized smooth endoplasmic reticulum," but with different intracellular localizations. Crockett et al.[35,36] discussed how the increased osteoclastogenesis from RANK signal sequence elongation remains unsolved, but offered interesting hypotheses.

2.8 Phenotype Variation of FEO, PDB2, ESH, PEBD, and JPD2

Why $FEO_{(Am)}$[16] is milder than $FEO_{(Ger)}$[17,18] and $FEO_{(NI)}$,[20–25] despite an identical duplication in *TNFRSF11A*[3] encoding RANK, remains unclear. Perhaps greater sunshine exposure, vitamin D levels, or dietary calcium intake diminished the severity of $FEO_{(Am)}$. There could also be different dental care or pharmacologic interventions. Significant gender influence, or left-right dominance conditioning where osteolysis appears, were not observed in $FEO_{(Am)}$.[16] However, trauma, illustrated by orthodonture that activates OCs to remove bone so that teeth can move, perhaps incites the osteolytic/expansile disease (see later).

In PDB, the marrow microenvironment is especially osteoclastogenic.[46] Paramyxovirus infection has been investigated as a pathogenetic factor (Chapter 25).[9,46] Indeed, OCs in $FEO_{(NI)}$ contain nuclear inclusions considered similar to PDB. However, PDB-like nuclear inclusions were identified in only 1 of 12 OCs in the lytic lesion studied in one $FEO_{(Am)}$ patient.[16] Two other $FEO_{(Am)}$ patients studied by electron microscopy had no OC inclusions. Perhaps the difference in severity between $FEO_{(Am)}$[16] and $FEO_{(NI)}$ or $FEO_{(Ger)}$ involves past viral infections. Nuclear inclusions have not been searched for in PDB2, ESH, PEBD, or JPD2. Nevertheless, FEO illustrates how focal skeletal lesions can be a major finding in a Mendelian metabolic bone disease.

The poor repair without osteosclerosis or hyperostosis during osteolytic expansion of an entire long bone in FEO compared to PDB is not understood. Although all bones are not equally impacted in FEO, extensive woven bone suggests that somehow the RANK mutation compromises OBs as well as OCs. Once the osteolysis has run its course in FEO, the expanded bone becomes fat-filled, perhaps because precursor mesenchymal stem cells are differentiating to adipocytes not OBs.[16] Understandably, advanced lesions are therefore unresponsive to antiresorptive therapy.[16]

The medical history of one $FEO_{(Am)}$ patient suggested that pregnancy (a time when skeletal turnover accelerates) can exacerbate the osteolytic disease.[16] Furthermore, the mother with ESH experienced hypercalcemia during lactation.[41] By extension, the effects of birth control pills or hormone therapy on the FEO skeleton merit investigation.

Perhaps trauma partly explains the focal nature of the osteolytic lesions seen in major long bones and the tooth destruction of FEO, and also the expanded phalanges in PDB2 and ESH. Indeed, trauma has been postulated to initiate osteolytic lesions in classic PDB.[47,48] Several individuals with $FEO_{(Am)}$ gave histories consistent with this possibility.[16] It may be that macroscopic or microscopic fractures in FEO initiate skeletal repair that becomes deranged because of excessive numbers and action of OCs. Osteolysis could then progress unchecked until the whole bone is involved, as in PDB.[9,47,48] In fact, evidence favoring trauma in the pathogenesis of the lysis of hard tissues in FEO includes the remarkable tooth loss that occurred in one young man with $FEO_{(Am)}$ who began orthodonture at 11 years-of-age.[16] Only he among his kindred suffered striking tooth loss. Bracing moves teeth by activating OCs within alveolar bone. In contrast, IER and tooth destruction were common years ago in $FEO_{(NI)}$, and therefore unlikely explained by orthodontic trauma. Furthermore, several individuals had iliac crest biopsies for $FEO_{(Am)}$ and did not develop osteolytic lesions.[16] Nevertheless, avoiding trauma and orthodonture (unless perhaps there is pharmacologic control of the disorder) seems prudent for FEO, PDB2, ESH, PEBD, and JPD2.

FEO varies considerably in "penetrance" (expression) from patient-to-patient and family-to-family with the 84dup18 mutation. Among the first reported kindreds, $FEO_{(Ger)}$[17,18] and $FEO_{(NI)}$[20–27] manifested severe polyostotic osteolytic/expansile disease and sometimes osteosarcomatous degeneration. In contrast, those with $FEO_{(Am)}$ developed only monoostotic disease, rarely had severe dental problems, and no bone malignancy was reported.[16] Importantly, Hughes et al.[3] confirmed by haplotype analysis that these first three reported FEO kindreds were not related, helping to explain how there could be interfamilial variations. Differences among affected individuals and kindreds despite identical RANK mutations would also likely reflect additional genetic, epigenetic, environmental, and so on, factors. $FEO_{(Sp)}$ showed that osteolytic/expansile bone disease could be uncommon in FEO, with deafness being the most prevalent complication.[30] Perhaps calcium and vitamin D sufficiency in $FEO_{(Am)}$ and $FEO_{(Sp)}$ helped restrain parathyroid hormone from further inciting any "riot" by the FEO OCs.[16] In 2011, van Hul[49] reviewed some of the genetic modifiers of monogenic bone diseases that we are now studying in FEO. Thus far, the duplications documented to cause constitutive activation of RANK would lead to four, five, six, and nine amino acid extensions of the signal peptide of RANK. Remarkedly, these different duplications seem to cause different dentoosseous phenotypes currently considered separate entities. PDB2 and ESH differ from FEO partly by their absence of focal osteolytic/expansile disease in major long bones (they manifest this instead in small tubular bones in the fingers), and by their episodic hypercalcemia.[11,37,41] In turn, PDB2 differs from ESH because the radiological findings of PDB2 in the adult years resembles classic polyostotic PDB (but with unusual osteosclerosis within the maxilla and widened mandible),[37,38] whereas ESH features a generalized skeletal disorder of hyperostosis and osteosclerosis.[11] PEBD causes shell-like bones diffusely.[8] JPD2 more closely resembles ESH and JPD.[13] Hence, FEO, PDB2, ESH, PEBD, and JPD2 are currently considered allelic (indeed "exonic") disorders of *TNFRSF11A* gain-of-function.

3 DISORDERS OF OPG DEFICIENCY

3.1 Juvenile Paget's Disease

Hyperphosphatasia refers to conditions that feature marked elevation of serum ALP activity (i.e., hyperphosphat*asemia*).[50] Among them are polyostotic fibrous dysplasia (including the McCune-Albright syndrome), Mabry syndrome (OMIM 239300)[10] also called hyperphosphatasia with mental retardation and due to impaired linkage of ALP to plasma membranes,[10] and entities that manifest focal or generalized acceleration of skeletal remodeling, such as the disorders of constitutive activation of RANK (see earlier), and JPD.[50] JPD has been called idiopathic or hereditary hyperphosphatasia (OMIM 23900).[10] Approximately 60 case reports of JPD are found in the medical literature.[5,51] Now, I believe at least three forms of JPD (JPD1, 2, and 3) can be discussed (see later).

JPD affects the entire skeleton. Understandably, objections arose to this designation because classic PDB had traditionally been regarded as a sporadic and focal skeletal disorder.[9,47,48] Now, however, revelations concerning family clustering and a genetic "diathesis" (i.e., predisposition) for PDB[9] and identification of the genetic basis for most JPD[51] has softened this objection (Chapter 25).

JPD1, due to OPG deficiency from loss-of-function mutations in *TNFRSF11B*, is the most common JPD and features the fastest rates of skeletal remodeling and the highest levels of BTMs encountered by clinicians. However, relatively mild forms have been described.[5,51] It is usually diagnosed in infancy or early childhood (Fig. 26.7).[5] JPD2 discussed earlier, from constitutive activation of RANK,[13] has been reported only once. JPD3 is distinctive and associated with mental retardation.[52] JPD1 is an AR condition, JPD2 is due to a heterozygous activating duplication in *TNFRSF11A* encoding RANK (see earlier),[13] and JPD3 occurs sporadically.[52]

JPD1 presents with bone pain, fracture, and deformity early in life.[51] Exceptionally, the skeletal disease

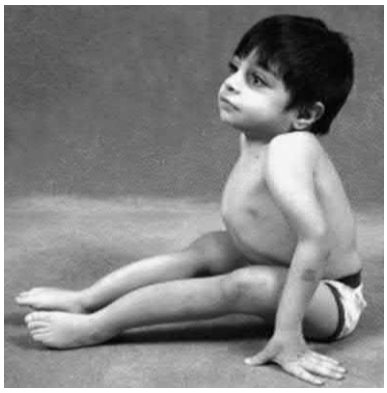

FIGURE 26.7 Physical findings in the osteoprotegerin (OPG) deficiency form of juvenile Paget's disease (JPD). This 4-year-old boy with untreated JPD has significant skeletal deformities due to homozygous loss-of-function mutation of *TNFRSF11B* encoding osteoprotegerin (OPG).

FIGURE 26.8 Physical findings in mild OPG deficiency JPD. (A) This 7-year-old girl of Puerto Rican heritage has relatively mild JPD from OPG deficiency. At age 2 years, she fractured her left proximal femoral diaphysis and later her left distal humerus, clavicle, and distal radius. (B) Note her broad forehead, leg-length inequality, lordosis, and flexion deformity of her knees. She has considerably milder JPD compared to the boy in Fig. 26.7 although homozygous for a loss-of-function (but different) mutation in *TNFRSF11B* encoding OPG.

is symptomatic during infancy, but usually diagnosis occurs in early childhood.[5] Relatively mild JPD1 causes fewer bony deformities and fractures (Fig. 26.8). Deafness and premature loss of teeth commonly occur in JPD1. However, the case reports that described the dental features preceded the discovery in 2002 that OPG deficiency from *TNFRSF11B* mutation causes JPD1 (see later). Hence, it is uncertain if these reports involved JPD1, though most probably do. Sometimes normal teeth were reported,[53–55] but most publications indicated missing incisors, delayed eruption of teeth,[56] loose

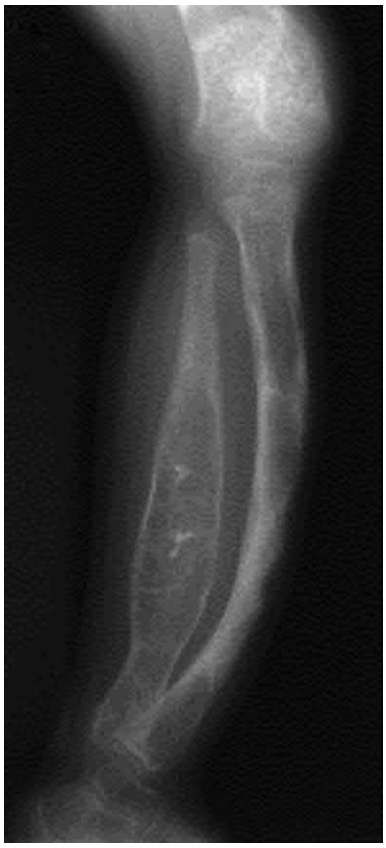

FIGURE 26.9 Radiographic findings in JPD. This lateral radiograph of the right leg of the boy at nearly 4 years-of-age, (Fig. 26.7) with untreated JPD has severe skeletal disease featuring expansion of the major tubular bones, areas of cortical thinning and thickening, and patchy areas of osteosclerosis and osteopenia that has led to bowing deformity. *Source: Reproduced from Whyte MP, Mumm S. Heritable disorders of the RANKL/OPG/RANK signaling pathway.* J Musculoskelet Neuronal Interact *2004;**4**:254–67, with permission.*

teeth, loss of lamina dura,[57] or premature shedding of deciduous teeth. In JPD1, radiographs show marked expansion (undertubulation) of long bones, initially with osteopenic cortices (Fig. 26.9). Eventually there is diffuse and acquired hyperostosis and osteosclerosis. The accelerated skeletal turnover markedly elevates BTMs, as supported by the histopathological findings in any bone.[51] The rapid remodeling causes "loss of chaos" of the trabecular bone pattern.[58] JPD1, PDB, and the disorders caused by *TNFRSF11A* activation can also manifest remarkable osteolytic/expansile defects. One such lesion appeared in a humerus of the Navajo proband with JPD despite PMD therapy (Fig. 26.10). Understandably, Chosich et al.[41] initially concluded that the mother with ESH had mild JPD (see earlier). However, mosaic bone characteristic of classic advanced PDB is absent in JPD. Measles virus transcripts, commonly reported in PDB,[59] have not been detected in peripheral blood leukocytes in mild[60] or severe cases

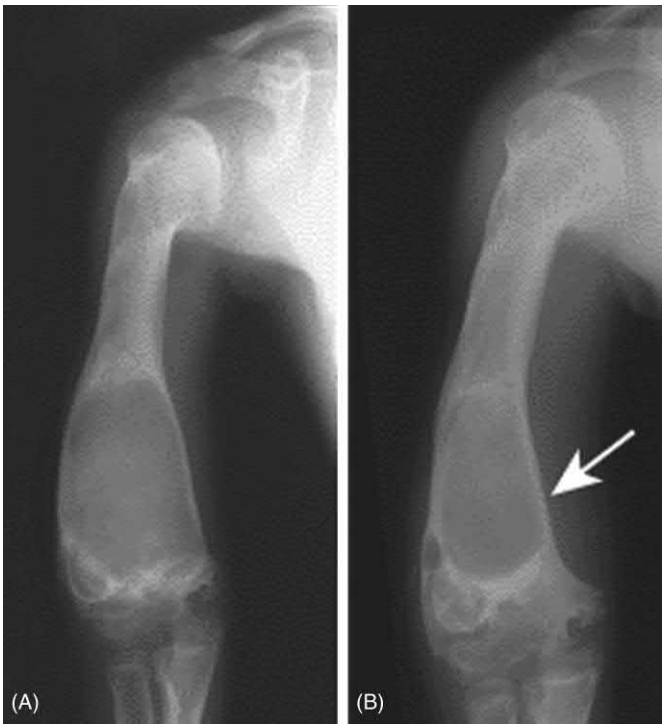

FIGURE 26.10 Osteolytic/expansile bone lesion in the OPG deficiency form of JPD. (A) This anteroposterior radiograph shows an expansile (soap bubble) lesion with cortical thinning in the distal humerus of a 7.5-year-old Navajo boy with JPD. It had been packed with autologous bone 9 months earlier, and a single intravenous infusion of pamidronate had been given 2 months previously. (B) At age 8.8 years-of-age, following additional infusions of pamidronate, the humerus has narrowed with improved modeling, and a bony cortex *(arrow)* has appeared. *Source: Reproduced from Whyte MP, Mumm S. Heritable disorders of the RANKL/OPG/RANK signaling pathway.* J Musculoskelet Neuronal Interact *2004;***4**:*254–67, with permission.*

of JPD (Whyte et al., unpublished). Fortunately, even JPD1 will respond to antiresorptive therapy, including human CTN[61] and various BPs, but patients will require treatment life long.[51] The genetic basis for JPD1 indicated that OPG replacement therapy would help these patients who have loss-of-function mutations within *TNFRSF11B*.[62] Although the anti-RANKL monoclonal antibody, denosumab, could help JPD1, a warning against sudden cessation of bone resorption came when zoledronic acid infusion caused significant hypocalcemia in one JPD1 patient.[63]

In 2002, homozygous deletion of *TNFRSF11B*, the gene that encodes OPG, was discovered in two Navajos with JPD.[5] They had identical break points on chromosome 8q24.2 encompassing selectively and completely this gene.[5] The deletion spanned ~100 kb, but the neighboring genes seemed intact. In keeping with this finding, the propositus' serum OPG and soluble RANKL levels were undetectable and markedly increased, respectively.[5] However, no *TNFRSF11B* or *A* mutation was detected in two unrelated women with relatively mild JPD, one representing JPD3.[5] That same year, Cundy et al.[64] identified a homozygous, in-frame, 3-bp deletion in *TNFRSF11B* in siblings of Iraqi ethnicity with JPD1. In a brief report in 2003,[12] we emphasized the importance of "founders" in the etiology of JPD1, showing different homozygous defects in *TNFRSF11B* in most, but not all, JPD patients that we could study worldwide. Absence of *TNFRSF11B* in Navajos with JPD1 likely reflected a heterozygous Navajo founder. Their population had decreased to ~6,000 people in 1,868, with this genetic "bottleneck" group then increasing to ~173,000 individuals in 2010.[65] Although the prevalence of this deletion among Navajos is not precisely known, we estimated ~1 in 100 to be heterozygous carriers.[66] Subsequently, similar evidence of different founders emerged from various geographical locations and "Balkan" and "Iberian" *TNFRSF11B* mutations were identified.[51] In 2003, Chong et al.[67] reported genotype–phenotype relationships for JPD1.

OPG is normally secreted into marrow spaces by cells derived from mesenchyme, including OBs.[1,2] AR loss-of-function mutations within *TNFRSF11B* impair biosynthesis of functional OPG and lead to excess uninhibited soluble and cell-surface RANKL that markedly accelerates bone turnover.[5] Although *Tnfrsf11b* knock-out mice were initially considered to have "osteoporosis," numerous OCs together with rapidly remodeling woven bone were present.[68] Accordingly, it might be said they manifest JPD. Heterozygous mice could be osteopenic.[68] Indeed, although the skeletal radiographs of the heterozygous parents of our three patients with JPD1 from different *TNFRSF11B* mutations were unremarkable (unpublished) despite approximately half-normal serum OPG levels, their DXA hip and spine BMD values ranged from average to just below the normal range (unpublished). Additional observations in JPD1 patients revealed an important role for OPG in preventing vascular microcalcification (VMC).[69] Although computed tomography of the aorta or renal arteries of a young Navajo woman with JPD showed no calcifications, VMC had been found on histopathological studies of the renal arteries and aorta of *Tnfrsf11b* knock-out mice.[69] The medical literature concerning JPD was particularly revealing. In 1971, in a report by Mitsudo,[57] all tissues obtained at autopsy from a 26-year-old man with JPD had striking changes consistent with pseudoxanthoma elasticum (OMIM 177850 and 264800),[10] including granular and coarse deposits of calcium in the membranes and intima of the muscular arteries and arterioles. In 1986, Silve et al.[70] reported a boy with JPD and "calcifying arteriopathy" detected by renal sonography and confirmed by histopathological examination of the internal elastic membrane of a temporal artery. In OPG deficiency, this VMC appears

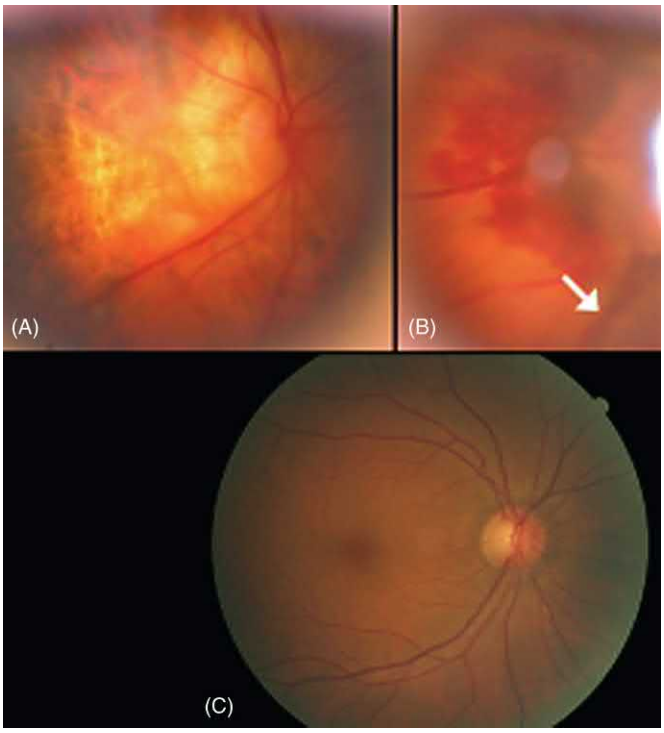

FIGURE 26.11 Retina findings in the OPG deficiency form of JPD versus FEO. In OPG deficiency JPD, vascular microcalcification (VMC) can cause retinopathy and blindness. Here, advanced retinal changes at 60 years-of-age include: (A) pigment changes and macular atrophy, (B) peripapillary subretinal hemorrhage with an angioid streak *(arrow)*, and in contrast (C), the retina of a 64-year-old woman with $FEO_{(Am)}$[16] is unremarkable. *Source: Reproduced from Whyte MP, Singhellakis PN, Petersen MB, Davies M, Totty WG, Mumm S.* J Bone Miner Res *2007;***22***:938–46, with permission.*

to cause retinopathy with blindness,[51,71] and perhaps explains cerebral aneurysms.[72] In contrast, the retina is unremarkable in FEO (Fig. 26.11). In 2010, the eye pathology of JPD1 was reviewed by Kerr et al.[71] Remarkedly, our Navajo proband with JPD also developed a brachial artery aneurysm at age 20 years that was successfully resected and contained bone (in manuscript). In 2016, we reported that auricular ossification is common in JPD1.[73] Renal ultrasound of the Navajo proband also revealed nephrocalcinosis together with tiny echogenic foci in his kidneys, perhaps representing small calculi,[5] but these calculi were likely due to his hypercalciuria. In fact, after PMD therapy, computed tomography of his kidneys reportedly became negative for calcifications (Whyte et al., unpublished).

JPD2 (see earlier) includes early-onset deafness, destruction of primary and secondary teeth, and a generalized skeletal disorder of accelerated bone turnover causing widened hyperostotic long bones that responded well to PMD treatment.[13] The etiology is a novel, sporadic, heterozygous, duplication (87dup15) in exon 1 of *TNFRSF11A* that would activate RANK.[3] Accordingly, there is genetic heterogeneity for the JPD phenotype, and AD forms seem possible.

JPD3 is especially rare, clinically more mild than JPD1, but features quite significant mental retardation.[52]

3.2 Acquired OPG Deficiency

Although not a Mendelian disorder, but of interest, in 2009 Riches et al. reported in women osteoporosis associated with neutralizing autoantibodies against OPG.[74] In fact, a clinical trial of a recombinant OPG may have been abandoned when antibodies to the biologic were detected. In 2015, autoantibodies to OPG were associated with increased bone resorption in rheumatoid arthritis.[75]

4 DISORDERS OF THE NF-κB COMPLEX

In 2016, Frederiksen et al.[14] reported a neonate with high bone mass who carried the first identified mutation of the five-member NF-κB complex. The defect was within *p65* encoding RELA.

5 DISORDERS OF RANKL AND RANK DEACTIVATION

In most OPTs, non-functional OCs are present in normal or elevated numbers.[6] In 2007, Sobacchi et al.[6] reported that AR loss-of-function mutations within *TNFSF11* encoding RANKL caused "OC-poor" OPT. Six such patients had few OCs and were not cured by hematopoietic stem cell transplantation.[6] In vitro, their OC precursor monocytes that had lacked endogenous RANKL stimulation responded to exogenous RANKL and formed functional OCs.[6]

In 2008, Guerrini et al.[7] reported a second form of "OC-poor" OPT in seven unrelated families. Here, AR loss-of-function mutations in *TNFRSF11A* encoding RANK kept monocytes from differentiating in vitro into OCs despite exposure to RANKL and M-CSF. This indicated an OC-intrinsic (cell-autonomous) defect. Such individuals also had hypogammaglobulinemia associated with impaired immunoglobulin-secreting B-cells.[7] Hematopoietic stem cell transplantation could rescue these patients.[7] In 2012, Pangrazio et al.[76] reported additional causal RANK mutations, and that the defect in the B-cell compartment occurred in only some patients but seemed to increase with aging.

However, there appears to be additional genetic heterogeneity for these "OC-poor" forms of OPT that seem to represent <10% of cases of AR OPT. In 2012,

in a preliminary communication,[77] we described a consanguineous toddler with "OC-poor" OPT who did not have a defect in *TNFSF11* encoding RANKL or *TNFRSF11A* encoding RANK. Indeed, it has been estimated that ~30% of cases of AR OC-poor OPT remain unexplained at the gene level.[76]

6 CONCLUSIONS

The Mendelian disorders that represent compromise within the RANKL/OPG/RANK/NF-κB signaling pathway are ultrarare and summarized in Fig. 26.12. Gain-of-function mutations in *TNFRSF11A* (RANK) and loss-of-function mutations in *TNFRSF11B* (OPG) cause generalized acceleration of bone turnover and its complications including deafness early in life from destruction of middle ear bones as well as loss of teeth. Thus far, *TNFRSF11A* activation of RANK by addition of amino acid extensions of various lengths to its signal peptide seems to cause distinctive disorders: FEO, PDB2, ESH, PEBD, and JPD2. Of interest, these disorders can manifest focal osteolytic and expansile bone lesions and resemble classic PDB despite their underlying germline mutations. Perhaps, local incitement of OC-mediated skeletal resorption from trauma explains the focal osteolysis. FEO when fully expressed causes osteolytic lesions that widen major long bones with pain, fracture, and deformity, and sometimes osteosarcoma, destruction of adult teeth, and deafness in childhood, and high-turnover osteoporosis. PDB2 resembles classic PDB, but also features maxillary and mandibular osteosclerosis, hypercalcemia from immobilization, and early-onset deafness and loss of adult teeth. ESH features accelerated bone remodeling, infancy-onset deafness, premature loss of teeth, hyperostotic widening of major long bones, painful expansile lesions within the phalanges of the hands but not major long bones, and episodic hypercalcemia. PEBD is most extraordinary featuring expanded shell-like bones. FEO, PDB2, ESH, PEBD, and JPD2 are "exonic" disorders caused by heterozygous, different length tandem duplications in exon 1 of *TNFRSF11A* that encodes the signal peptide of RANK. Transfection studies indicated proteolytic cleavage of the elongated RANK signal sequence is impaired, trapping each receptor intracellularly and somehow causing RANK gain of function. AR loss-of-function defects within *TNFRSF11B* causes OPG deficiency, and most occurrences of JPD (JPD1) reflect founder mutations. JPD1 features the highest rates of bone remodeling and leads to childhood deafness, tooth loss, fractures, bone deformities, and uniquely to auricular ossification and VMC that causes retinopathy and blindness, and perhaps arterial aneurysms. In stark

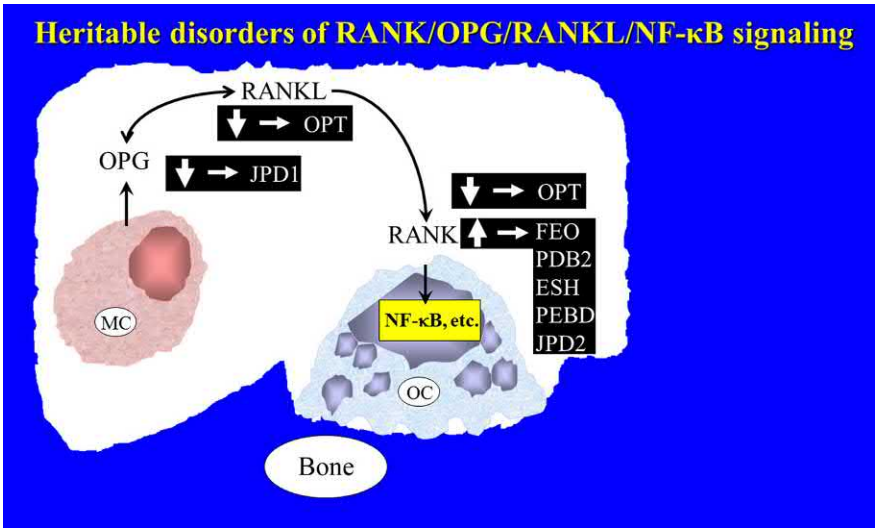

FIGURE 26.12 Mendelian disorders of RANKL/OPG/RANK signaling. In familial expansile osteolysis (FEO), early-onset Paget's disease of bone (PDB2), expansile skeletal hyperphosphatasia (ESH), panostotic expansile bone disease (PEBD), and juvenile Paget's disease, type 2 (JPD2), constitutive activation of RANK enhances osteoclastogenesis due to tandem duplications of different lengths in exon 1 of *TNFRSF11A*. These duplications extend the signal peptide for RANK trapping it intracellularly. In most patients with juvenile Paget's disease (JPD1), homozygous loss-of-function mutations in *TNFRSF11B* that encodes OPG causes OPG deficiency leading to enhanced RANK activity. In contrast, loss-of-function mutations in *TNFSF11* encoding RANKL or in *TNFRSF11A* encoding RANK cause osteopetrosis (OPT) featuring deficiency of osteoclasts (OCs). To date, activating mutations in *TNFSF11* or *TNFRSF11B* have not been reported, but should be sought among the unexplained "hyperphosphatasia" and OC-poor OPTs, respectively. The first mutation within the five-member NF-κB complex, a loss-of-function defect in *p65* (RELA), was associated with neonatal osteosclerosis. *Source: Updated from Whyte MP, Mumm S. Heritable disorders of the RANKL/OPG/RANK signaling pathway.* J Musculoskelet Neuronal Interact *2004;***4***:254–67, with permission.*

contrast, AR loss-of-function mutations in *TNFSF11* (RANKL), and *TNFRSF11A* (RANK) impair osteoclastogenesis and lead to especially rare "OC-poor" forms of OPT. Gain-of-function mutations in *TNFSF11* or *TNFRSF11B* have not been reported, but might explain some "hyperphosphatasia" and OC-poor OPTs, respectively. The first Mendelian disorder involving one of the five members of the NF-κB complex (RELA) was reported in 2016 and features neonatal osteosclerosis probably due to diminished OC action together with increased OB action.

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ABBREVIATIONS

AD Autosomal dominant
ALD Alendronate
ALP Alkaline phosphatase
AR Autosomal recessive
BP Bisphosphonate
BTM Bone turnover marker
CT Computed tomography
CTN Calcitonin
EHDP Etidronate
ESH Expansile skeletal hyperphosphatasia
FEO Familial expansile osteolysis
IER Idiopathic external resorption
JPD Juvenile Paget's disease
NF-κB Nuclear factor-kappa B
OB Osteoblast
OC Osteoclast
OHP Hydroxyproline
OPG Osteoprotegerin
OPT Osteopetrosis
PDB Paget's disease of bone (late-onset)
PDB2 Early-onset PDB
PMD Pamidronate
RANK Receptor activator of NF-κB
RANKL RANK ligand
TNF Tumor necrosis factor
TNFSF11 TNF superfamily, member 11
TNFRSF11A TNF receptor superfamily, member 11A
TNFRSF11B TNF receptor superfamily, member 11B
VMC Vascular microcalcification

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CHAPTER

27

Skeletal Dysplasias

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1 INTRODUCTION

The skeletal dysplasias are a diverse group of genetic disorders of the skeleton, which usually manifest during pre- and postnatal growth. There are over 400 recognized types of dysplasias. They produce a wide variety of phenotypes, including short stature, skeletal deformities, and increased bone fragility.

Each type of skeletal dysplasia is rare but, overall, the worldwide incidence is approximately 1:5000–1:3000 births.[1,2] Patients with skeletal dysplasias are best managed by specialized multidisciplinary clinics and services that have experience in diagnosis and management of these rare disorders. Skeletal dysplasia clinics are usually headed by a clinical geneticist and can include a wide range of physicians, surgeons, genetic counselors, and allied health professionals, as well as laboratory and research staff.

Establishing a firm diagnosis is the initial focus of the skeletal dysplasia clinic team. It is very important that children with suspected skeletal dysplasia are promptly referred to a specialized center, so that appropriate advice can be provided about the likely diagnosis, investigations, genetic testing, recurrence risks, current clinical problems, expected clinical course, and treatments. Prompt referral of the parents is also recommended whenever a fetus is suspected to have a skeletal dysplasia, whether it is during the pregnancy or following a stillbirth. Establishing the diagnosis and, where appropriate, the molecular diagnosis, is often critical in planning and managing future pregnancies. Adults who are referred with a possible skeletal dysplasia need to locate their childhood radiographs, as many of the diagnostic radiographic features are lost after skeletal maturity. Unfortunately, these radiographs have often been discarded.

Over the past decade, there has been an exponential increase in the number of genetic variants associated with skeletal dysplasias. Even more rapid progress can be expected in the future because of the widespread use of novel DNA-sequencing technologies, including exome and whole-genome sequencing. These technologies not only facilitate the identification of novel skeletal dysplasia genes, but also make genetic testing more cost effective. This therefore allows the testing of more patients for mutations, thus expanding the mutational spectrum and allowing for better genotype–phenotype correlations.

This chapter provides a summary of the current classification of the skeletal dysplasias, key points about making a diagnosis, and, finally, descriptions of the multiple epiphyseal dysplasias and the metaphyseal dysplasias. The latter dysplasias were selected because they are typical examples of skeletal dysplasias.

2 CLASSIFICATION OF SKELETAL DYSPLASIAS

Commencing in the 1960s, the nomenclature of the skeletal dysplasias has undergone numerous revisions as new clinical and molecular information has become available. The International Skeletal Dysplasia Society (ISDS), which was established in 1999, is responsible for the current revisions. The latest revision, published in 2015, includes over 400 different conditions, which are placed into over 40 groups defined by molecular, biochemical, and radiographic criteria.[3] The classification is summarized in Table 27.1.

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TABLE 27.1 Classification of Skeletal Dysplasias

Group no.	Group name	Group no.	Group name
1.	FGFR3 chondrodysplasia group	2.	Type 2 collagen group
3.	Type II collagen group	4.	Sulfation disorders group
5.	Perlecan group	6.	Aggrecan group
7.	Filamin group and related disorders	8.	TRPV4 group
9.	Ciliopathies with major skeletal involvement	10.	MED and pseudoachondroplasia group
11.	Metaphyseal dysplasias	12.	SMD
13.	SE(M)D	14.	Severe spondylodysplastic dysplasias
15.	Acromelic dysplasias	16.	Acromesomelic dysplasias
17.	Mesomelic and rhizomesomelic dysplasias	18.	Campomelic dysplasia and related disorders
19.	Slender bone dysplasia group	20.	Dysplasias with multiple joint dislocations
21.	CDP group	22.	Neonatal osteosclerotic dysplasias
23.	Osteopetrosis and related disorders	24.	Other sclerosing bone disorders
25.	Osteogenesis imperfecta and decreased bone density group	26.	Abnormal mineralization group
27.	Lysosomal storage diseases with skeletal involvement (dysostosis multiplex group)	28.	Osteolysis group
29.	Disorganized development of skeletal components group	30.	Overgrowth syndromes with skeletal development
31.	Genetic inflammatory/rheumatoid-like osteoarthropathies	32.	Cleidocranial dysplasia and related disorders
33.	Craniosynostosis syndromes	34.	Dysostoses with predominant craniofacial involvement
35.	Dysostoses with predominant vertebral with or without costal involvement	36.	Patellar dysostoses
37.	Brachydactylies (without extraskeletal manifestations)	38.	Brachydactylies (with extraskeletal manifestations)
39.	Limb hypoplasia: reduction defects group	40.	Ectrodactyly with and without other manifestations
41.	Polydactyly–syndactyly–triphalangism group	42.	Defects in joint formation and synostoses

CDP, Chondrodysplasia punctata; FGFR3, fibroblast growth factor receptor 3; MED, multiple epiphyseal dysplasia; SE(M)D, spondyloepi(meta)physeal dysplasias; SMD, spondylometaphyseal dysplasias; TRPV4, transient receptor potential cation channel subfamily V member 4.

The ISDS Classification initially grouped disorders by clinical–radiographical similarities. As more of the underlying genetic defects were discovered, attempts were made to group disorders by affected genes. The result is a hybrid classification in which some disorders are grouped by gene and others by clinical–radiographic features, which sometimes requires cross-referencing between groups. Currently, groups 1–9 are defined by their molecular characteristics, and groups 9–42, are defined by their clinical and radiological phenotypes. The classification is in a state of transition. As the remaining skeletal dysplasia genes are being identified and a better understanding of the pathogenetic mechanisms underlying these disorders is gained, grouping disorders by the molecular mechanism of disease will become more useful. On the other hand, grouping disorders by clinical characteristics still has its merit for facilitating diagnosis. It is likely that two complementary classifications of skeletal dysplasias will emerge: one using the clinical phenotypes and the other using the molecular characteristics.

3 DIAGNOSIS

There are two broad types of clinical presentations of patients, usually children, to skeletal dysplasia clinics. One presentation is where the proband comes from a family with a known skeletal dysplasia. It is necessary to determine whether the proband is affected or not and to confirm that the diagnosis is correct. The other presentation is where the proband's family is not known to have a skeletal dysplasia. In this case, it is necessary to determine whether the proband and other family members have a skeletal dysplasia and if so the type of dysplasia.

Families with diagnosed skeletal dysplasias may already be registered with the referral skeletal dysplasia clinic or, if not, the relevant information needs to be

obtained from other clinics. Detailed review of all available information about the index patient and other affected family members is usually undertaken before the index individual is reviewed in the clinic. It is also helpful if other affected members of the family attend the initial consultation, as additional information is often needed concerning the diagnosis and clinical course.

Thorough assessment of the index individual involves detailed prenatal, perinatal, postnatal, and family histories, as well as detailed general and musculoskeletal examinations. The family needs to bring growth charts or a list of measurements of length, height, weight, and head circumference that can be plotted on the appropriate charts. For some skeletal dysplasias, diagnosis-specific growth charts are available.[3a–c]

Some skeletal dysplasias produce typical dysmorphic clinical features that are instantly recognizable to expert clinicians. Achondroplasia, with its rhizomelic short stature and characteristic craniofacial and hand features, is an example of a relatively common skeletal dysplasia that is easily diagnosed clinically. However, the majority of skeletal dysplasias cannot be readily diagnosed from their external appearance. In either event, it is prudent to confirm a clinical diagnosis through further evaluations and investigations, as errors in clinical diagnosis can have significant implications for genetic counseling and management.

Diagnostic imaging is an essential requirement in the evaluation of children with a suspected skeletal dysplasia. A complete skeletal survey is recommended and should include plain radiographs of the skull (lateral view), spine (anterior-posterior and lateral view), thorax (anterior-posterior view), pelvis (anterior-posterior view, which should include the lower lumbar spine and the proximal femurs), both knees (anterior-posterior view) and both hands (anterior-posterior view). Depending on the diagnoses considered, additional radiographs might be required. Detailed evaluations of the size, shape, and structure of each bone are undertaken. The findings are often summarized in accordance with the predominant pattern of changes in the skeleton, such as spondylo, epiphyseal, metaphyseal, and diaphyseal abnormalities. These terms may be grouped together to reflect the diversity of skeletal phenotypes, for example, multiple epiphyseal dysplasia, spondyloepiphyseal dysplasia, and spondyloepimetaphyseal dysplasia. Other patterns may involve changes in the density and shape of bones of the appendicular and axial skeleton.

In the hands of an expert, the clinical and radiographic features are usually sufficient to enable a diagnosis or differential diagnosis to be made. Most radiologists, clinical geneticists, and orthopedic surgeons have insufficient exposure to these rare disorders to confidently make a diagnosis. In these cases, the expert opinion from a skeletal dysplasia center should be sought. It is important to note that the radiographic features often change with growth. In some cases, the typical radiographic features may need time to develop while in others, they may disappear as the patient gets older. Many radiographic features get lost following skeletal maturity. Consequently, adults should bring their childhood radiographs to their initial consultation, as they may provide valuable diagnostic information concerning epiphyseal, physeal, and metaphyseal growth abnormalities. Similarly, repeated skeletal surveys are often useful in establishing a diagnosis in a child when the initial clinical and radiographic evaluation is inconclusive.

Magnetic resonance imaging (MRI) and computerized tomography (CT) are rarely used to establish the diagnosis of a skeletal dysplasia. However, they are often used to evaluate specific problems that occur frequently in certain types of dysplasias. For example, MRI is often used to evaluate cervical spinal cord compression in patients with achondroplasia or various forms of spondyloepiphyseal dysplasia, as a result of stenosis of the foramen magnum or instability of the atlanto–axial joints. MRI and CT, as well as MRI and CT angiograms, are often used for preoperative planning for a surgical removal of exostoses in patients with various types of multiple hereditary exostosis.

Prenatal ultrasonography is a routine part of medical care during pregnancy. Short limbs for gestational age and polyhydramnios may indicate an underlying skeletal dysplasia. Once a skeletal dysplasia is suspected the patient should be referred to a specialized center for a detailed morphological assessment by ultrasonography. Accurate prenatal ultrasound diagnosis of skeletal dysplasias remains difficult despite advances in ultrasound technology.[3d,3e]

Fetal CT with 3D reconstruction allows for excellent visualization of the fetal skeleton and can be a valuable tool in the prenatal assessment of a fetal skeletal dysplasia.[4] Concerns over exposing the fetus to ionizing radiation have limited the use of technique, but with modern protocols and equipment, this should no longer be a barrier. However, interpretation of these images still requires expertise in skeletal dysplasias and should therefore be carried out in liaison with a skeletal dysplasia center.

Genetic testing during pregnancy is difficult, as results have to be obtained very quickly to be useful for pregnancy management. Results are also more difficult to interpret, as the fetus cannot be properly examined. Genetic testing is therefore usually limited to pregnancies in which a specific diagnosis is strongly suspected. Bone dysplasia gene panels, based on high-throughput sequencing technology, have recently become available and can test for dozens of bone dysplasia genes in a single test. The clinical utility of these tests in the prenatal setting still needs to be confirmed. Otherwise, molecular testing is undertaken following delivery or termination of the pregnancy.

Biochemical investigations are helpful in children with suspected rickets, mucopolysaccharidoses, mucolipidoses, or chondrodysplasia punctata. These investigations, coupled with molecular diagnosis, are undertaken as soon as these dysplasias are suspected, so that appropriate treatments (if available) can also be commenced early, when they are most effective.

Histology of autopsy material from lethal skeletal dysplasias can be an adjunct to radiographs in establishing a clinical diagnosis. Sections through the growth plates are the most helpful in this regard. Postnatal iliac crest cartilage biopsy has limited value in the diagnosis of chondrodysplasias, but quantitative histomorphometry of iliac crest bone is routinely undertaken in many centers to monitor bisphosphonate therapy of patients with osteopenia due to osteogenesis imperfecta and related conditions. Histological studies of excised tissue from dysplasias that predispose individuals to malignancy, for example, multiple hereditary exostosis, is routine. Bone marrow histology is also undertaken in skeletal dysplasias that are associated with bone marrow anomalies, including predisposition to malignancies, such as leukemias and lymphomas. Examples include patients with cartilage–hair hypoplasia (CHH) or Shwachman–Bodian–Diamond syndrome (SBDS).

Molecular diagnosis is important for the confirmation of the clinical and radiological diagnoses of a skeletal dysplasia. It is valuable in confirming inheritance patterns and risks of recurrence, as well as for the planning of pregnancies. Molecular diagnosis has also rapidly expanded the amount of genetic information concerning skeletal dysplasias and the wide spectrum of genes involved in normal skeletal development. In some cases, the molecular advances have resulted in specific therapies. For example, a drug that specifically targets the enchondral ossification pathways inhibited by the classic achondroplasia mutation (fibroblast growth factor receptor 3 or *FGFR3*) is currently in clinical trials. In mucopolysaccharidoses, enzyme replacement therapy, by the administration of exogenous enzyme or by endogenous production from transplanted allogeneic bone marrow hemopoietic stem cells, improves many of the clinical symptoms of these disorders though, unfortunately, the effect on the skeletal features is less pronounced. In contrast, the bone phenotype and bone marrow function improves in some children, with severe forms of osteopetrosis treated by allogeneic bone marrow hemopoietic stem cell transplantation.

Therapies for some of the other skeletal dysplasias involve pharmacological modulation of physiological processes that are impaired because of the mutations; for example, the administration of phosphate and vitamin D to patients with various forms of genetic rickets and the administration of bisphosphonates to decrease the abnormally high levels of bone turnover in many forms of osteogenesis imperfecta.

Unfortunately, comprehensive information on skeletal dysplasias is not widely available. GeneReviews and OMIM, two popular online encyclopedias on genetic disorders, contain entries for the more common skeletal dysplasias, but not for the rarer ones. The same is true for diagnostic software packages commonly used in clinical genetics, such as POSSUM and London Dysmorphology Database. The Skeletome Knowledge Base (knowledge.skeletome.org) is a new resource that aims to provide expert-curated summaries on *all* bone dysplasias, but is still under development. There are a number of good bone dysplasia atlases that provide succinct summaries and example radiographs that can help with diagnosis.[4a,5] However, use of these books still requires significant expertise. Detailed information on long-term natural history or management of specific complications is sparse. Limited information can be found in the medical literature. However, many studies suffer from small case numbers, insufficient clinical detail, or even misdiagnosis in the cases that are included. Due to these issues, patients are best managed by bone dysplasia centers that have the required expertise. Many bone dysplasia centers provide diagnostic and management advice over the internet if a direct patient visit is not practical. An example is the European Skeletal Dysplasia Network (www.esdn.org), which allows the submission of clinical summaries and radiographs via its website for review by an expert committee.

4 MULTIPLE EPIPHYSEAL DYSPLASIAS

The multiple epiphyseal dysplasias (Table 27.2) are a relatively common group of conditions that are included within groups 4, 6, and 10 of the 2015 revision of the classification of genetic skeletal disorders. They are characterized by abnormal development of the epiphyses of the appendicular skeleton (Fig. 27.1) with mild or no visible changes in the axial skeleton.

Children with the autosomal dominant (AD) forms appear to be normal at birth, but develop slowing of longitudinal growth, painful hips and knees, altered gait, and genu valgum in early childhood. At initial presentation, these changes may be quite mild. A skeletal survey typically reveals delayed and abnormal development of multiple upper and lower limb epiphyses. Most appendicular epiphyses are small and may be misshapen. The femoral heads may not only show similar changes, but may also show multiple small centers of ossification, which coalesce in late childhood and adolescence. This often leads to an incorrect diagnosis of Perthes disease, a form of avascular necrosis, of the hip. A skeletal survey is essential because of the difficulty in distinguishing

TABLE 27.2 Multiple Epiphyseal Dysplasias

Names of disorders	Modes of inheritance	Genes	Proteins
EDM1	AD	*COMP*	Cartilage oligomeric matrix protein
EDM2	AD	*COL9A2*	Collagen 9, α2 chain
EDM3	AD	*COL9A3*	Collagen 9, α3 chain
EDM4	AR	*DTDST*	SLC26A2 sulfate transporter
EDM5	AD	*MATN3*	Matrilin 3
EDM6	AD	*COL9A1*	Collagen 9, α1 chain
MED other types	AD		
Familial osteochondritis dissecans	AD	*AGC1*	Aggrecan
Stickler syndrome, recessive type	AR	*COL9A1*	Collagen 9, α1 chain
Familial hip dysplasia (Beukes)	AD	*UFSP2*	Ubiquitin-fold modifier 1–specific peptidase 2
Multiple epiphyseal dysplasia with microcephaly and nystagmus (Lowry–Wood)	AR		

AD, Autosomal dominant; AR, autosomal recessive; EDM, epiphyseal dysplasia, multiple.

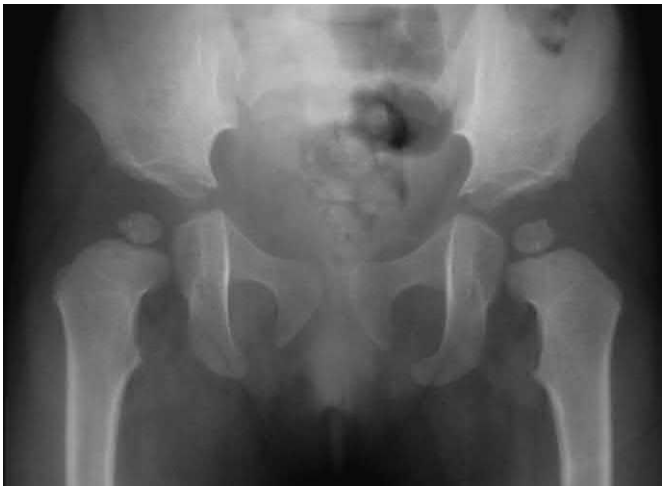

FIGURE 27.1 Pelvic radiograph of a 4-year-old child with AD EMD1 due to a missense mutation in *COMP*. The epiphyses are irregular and underdeveloped. The acetabulum also shows dysplastic changes.

Perthes disease from multiple epiphyseal dysplasia if radiological studies are limited to the pelvis and hips. This distinction is also important because some patients with well-defined types of multiple epiphyseal dysplasia may develop secondary Perthes disease in one or both hips during childhood. Likewise, multiple epiphyseal dysplasia can be mistaken for osteochondritis dissecans (see later) if no full skeletal survey is obtained. The vertebrae are often normal, but may be ovoid with mild irregularity of the vertebral end plates.

Table 27.2 lists the known genes associated with AD forms of multiple epiphyseal dysplasia. They include the genes encoding cartilage oligomeric matrix protein (COMP), matrilin 3, and the three α-chains of type IX collagen.[6–10] Although their clinical and radiological features are similar, there are also some differences. For example, joint laxity and a mild myopathy are found in those with *COMP* mutations (multiple epiphyseal dysplasia type 1 or EMD1) related to the expression of COMP protein in ligaments, tendons, and muscles, as well as in hyaline cartilage and bone. Patients with EMD1 also have ovoid vertebral bodies and mild irregularity of the vertebral end plates. The clinical and radiological features of EMD1 are similar, but milder, than those of pseudoachondroplasia (PSACH), which is a form of spondyloepimetaphyseal dysplasia also caused by mutations of *COMP*. Muscular weakness, usually without the joint laxity of EMD1, is also observed in some patients with mutations of *COL9A2* and *COL9A3*.[11] In the latter patients, the muscle weakness involves the proximal muscles of the limbs. Mouse studies have shown that muscle weakness is likely to be due to an underlying tendinopathy that is more pronounced patients with *COMP* mutations than those with *MATN3* mutations.[12,13]

The radiographic features of EMD2, due to mutations of *COL9A2*, can mimic the changes of osteochondritis dissecans.[11] These changes can also occur in familial osteochondritis dissecans due to an AD mutation of *AGC1*, which encodes aggrecan.[14] An example of a patient with features of epiphyseal dysplasia and osteochondritis dissecans is shown in Fig. 27.2.

All forms of AD multiple epiphyseal dysplasia are associated with progressive skeletal impairments. Some patients develop progressive genu valgum, which may require surgical correction. Progressive osteoarthritis, particularly of the hips and knees, is common. Total joint

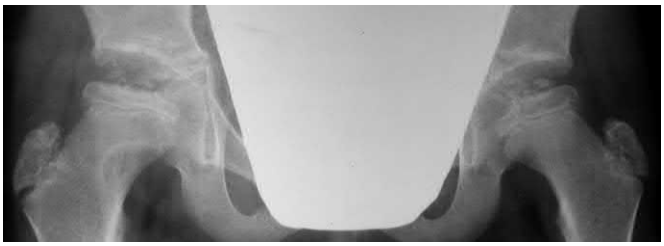

FIGURE 27.2 Pelvic radiograph of an 8-year-old child with AD mixed MED and osteochondritis dissecans phenotype due to an *AGC1* mutation. The secondary ossification centers of the femoral heads are flattened and irregular. At skeletal maturity, there was a persistent area of central osteochondritis dissecans in each femoral head.

replacements of hips, knees, or both are often needed in the fourth decade but may be needed earlier, particularly in those with severe deformities of the femoral heads at skeletal maturity.

EMD4 or rMED is the only autosomal recessive (AR) variant (Fig. 27.3). Patients with EMD4 are frequently born with club feet and later develop clinical and radiological features of multiple epiphyseal dysplasia.[15] Some patients also have recurrent subluxation and dislocation of the patellae. Homozygous or compound heterozygous mutations of the *DTDST* (*SLC26A2*) gene, which encodes a sulfate transporter, have been identified in patients with EMD4. The femoral heads are often severely affected and may produce rapidly progressive osteoarthritis requiring total hip joint replacements in the second decade. A double-layered patella is common in patients with EMD4. It is noted on lateral radiographs of the knee, as a result of separate anterior and posterior centers of ossification (Fig. 27.3). However, the patella findings are not confined to patients with EMD4 because similar radiographic findings have been observed in patients with pseudoachondroplasia due to AD mutations of *COMP* and in EMD2 due to AD mutations of *COL9A2*.[16,17]

There is a significant overlap in the clinical and radiological findings among the epiphyseal dysplasias. Genetic testing for the epiphyseal dysplasias should therefore best be performed through a gene panel that includes all of the currently known epiphyseal dysplasia, multiple (EDM) genes (COMP, MATN3, COL9A1, COL9A2, COL9A3, and SLC26A2). Ideally, the genes for osteochondritis dissecans and other overlapping phenotypes should also be included in such a panel, while testing of COL2A1 may also be warranted for nontypical MED variants.[18] It has been shown that expert clinical review can more than double the mutation detection rate,[19] thus expert clinical review should be sought wherever possible before embarking on genetic testing. Importantly, even after expert review, no mutation is identified in ~20% of cases of MED, suggesting that additional genes for MED still await discovery. These cases are classified as MED other types (Table 27.2).

5 METAPHYSEAL DYSPLASIAS

The metaphyseal dysplasias are listed in Table 27.3 and are included within group 11 of the 2015 revision of the classification of genetic skeletal disorders. These dysplasias have radiographic evidence of abnormal metaphyses, often with cupping and irregularity due to the persistence of cartilage columns in the metaphyseal bone. However, the primary cause of the latter changes usually resides in the physis or growth plate. The metaphyseal dysplasias included in Table 27.3 involve the appendicular skeleton and have mild or no apparent anomalies in the axial skeleton. Dysplasias with more severe spinal and epiphyseal changes are referred to as spondylometaphyseal and spondyloepimetaphyseal dysplasias, respectively, and are listed elsewhere in the ISDS nosology.

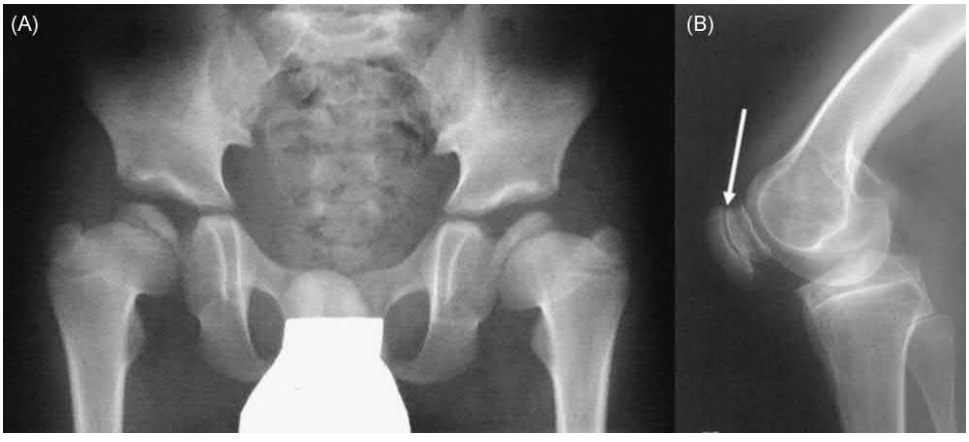

FIGURE 27.3 AR EMD4 due to mutations of *DTDST*. (A) Pelvic radiograph at the age of 12 years shows flattened and small secondary centers of ossification of the femoral heads. (B) Lateral radiograph of knee at 17 years of age showing a double-layered patella.

TABLE 27.3 Metaphyseal Dysplasias Group

Names of disorders	Modes of inheritance	Genes	Proteins
MCS	AD	*COL10A1*	Collagen X, α1 chain
CHH	AR	*RMRP*	RNA component of RNAse H
Metaphyseal dysplasia, Jansen type	AD	*PTHR1*	PTH/PTHrP receptor 1
Eiken dysplasia	AR	*PTHR1*	PTH/PTHrP receptor 1
Metaphyseal dysplasia with pancreatic insufficiency and cyclic neutropenia (SBDS)	AR	*SBDS*	SBDS protein
Metaphyseal anadysplasia type 1	AD/AR	*MMP13*	Matrix metalloproteinase 13
Metaphyseal anadysplasia type 2	AR	*MMP9*	Matrix metalloproteinase 9
Metaphyseal dysplasia, Spahr type	AR	*MMP13*	Matrix metalloproteinase 13

AD, Autosomal dominant; AR, autosomal recessive; CHH, cartilage–hair hypoplasia; MCS, metaphyseal dysplasia, Schmid type; SBDS, Shwachman–Bodian–Diamond syndrome.

Children with metaphyseal dysplasia typically present either at birth or after they start to stand and walk.

Children with the most common form of metaphyseal dysplasia, the Schmid type, typically present with bow legs that develop after the children start to stand and walk. The children are usually considered to be normal at birth. A family history of skeletal dysplasia may be present. In any event, a careful history, examination, skeletal survey, and biochemical tests for rickets are required. There is an extensive differential diagnosis. Physiological bow legs are the most common type of bow legs in infancy. The latter children have normal height and growth, their skeletal survey shows radiographic changes confined to the knees, and their biochemistry tests are normal. Physiological bow legs resolve over the following few years. Another common cause of bow legs in infancy is Blount's disease, which is a progressive form of tibia vara. The children are often obese, their height is normal, their skeletal survey shows radiological changes confined to the knees, and their biochemical tests are normal. The next common cause of bow legs in infancy is rickets. Children with nutritional and genetic types of rickets show impaired longitudinal growth, widespread widening, irregularity of growth plates, cupping of metaphyses, bowing of long bones, osteopenia, and abnormal biochemical tests for rickets. The genetic forms of rickets are included in group 26, the abnormal mineralization group, of the 2015 revision of the classification of genetic skeletal disorders.

Children with AD metaphyseal dysplasia, Schmid type (MCS), are apparently normal at birth. Progressive bowing appears in early childhood and growth slows from the age of walking. There are widespread changes in the growth plates of the limbs with the most severe changes being in the hips and knees (Fig. 27.4). The growth plates are widened as in rickets and the metaphyses are irregular due to the persistence of columns of chondrocytes within the trabecular bone. The long bones in the legs are bowed but appear to have normal bone density. The medial cortices of the bowed long bone diaphyses are markedly thickened. Biochemical tests for rickets are normal. Mutational analyses show that children with MCS are usually heterozygous for mutations of

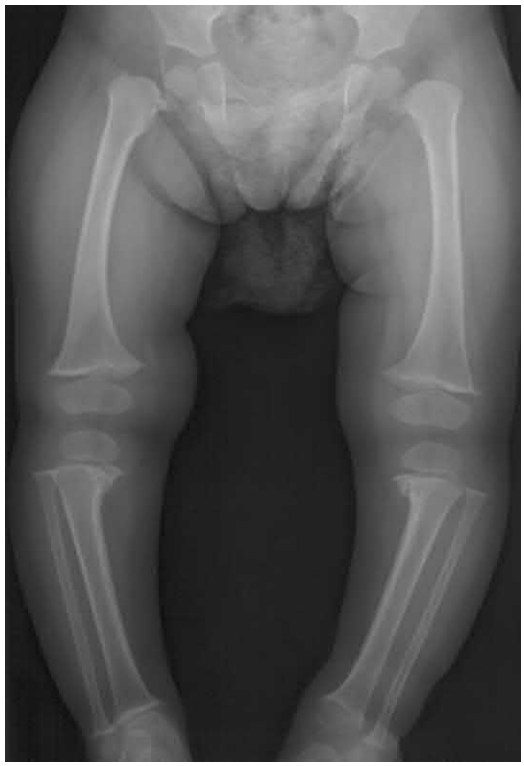

FIGURE 27.4 Radiograph of the pelvis and legs in a 2-year-old child with AD metaphyseal dysplasia, Schmid type, due to a frameshift mutation of *COL10A1*. The radiographs show metaphyseal abnormalities in femurs and tibias.

COL10A1, which encodes the α1(X) chain of collagen X. The expression of *COL10A1* is confined to the hypertrophic chondrocytes of the growth plate. The mutations are largely confined to the noncollagenous, carboxy-terminal domain (NC1) of α1(X) chains and include nonsense mutations, frameshift mutations, and missense mutations.[20] Only two mutations have been identified at the amino-terminal signal peptide cleavage site, and these would prevent cleavage and release of the α1(X) chains into the lumen of the endoplasmic reticulum, resulting in a deficiency of collagen X secretion.[21] There has been one reported Gly substitution mutation in the collagen X triple helix,[22] but the pathogenicity of this is unclear, given the large number of helix Gly substitutions reported in normal individuals in the Exome Aggregation Consortium (ExAC) database (http://exac.broadinstitute.org).

Nonsense mutations or frameshift mutations that produce premature termination codons yield mutant mRNAs that undergo variable amounts of nonsense-mediated decay. In patients with complete loss of the mutant mRNA, presumably from nonsense-mediated decay, the metaphyseal dysplasia has a late-onset in midchildhood.[22a,22b] This finding is in keeping with the mild and late-onset phenotype associated with the complete lack of collagen X in *Col10a1*-null mice. Patients with partial loss of mutant mRNA, due to nonsense-mediated decay, have an early-onset of metaphyseal dysplasia when the children commence standing and walking.[22b,22c] The biochemical findings suggest that the remaining truncated α1(X) protein chains are able to impair the formation of normal collagen X molecules. Mice bearing similar mutations also show early expression of a progressive form of metaphyseal dysplasia. The reduced amounts of collagen X in the matrix of the hypertrophic zone, due to nonsense-mediated decay and impaired secretion of abnormal collagen X molecules, may play a part in the pathogenesis of the metaphyseal dysplasia.

Missense mutations of the NC1 domain of collagen X have been shown to impair the assembly of collagen X trimers and compromise secretion. Due to the extracellular pathological consequence of reducing collagen X in the extracellular matrix, the misfolded mutant collagen X retained within the rough endoplasmic reticulum stimulates an unfolded protein stress response.[23] This unfolded protein response triggers a number of cellular mechanisms in an attempt to restore proteostasis by increasing chaperone synthesis to improve folding, reduce protein translation, and stimulate proteasomal mutant collagen X degradation. However the persistent unfolded protein response triggers signaling pathways that impair terminal differentiation of the hypertrophic chondrocytes[24] and leads to the persistence of pre-hypertrophic and hypertrophic chondrocyte columns in the metaphysis. Induction of an unfolded protein stress response in hypertrophic chondrocytes of mice, using a collagen X promoter to drive the expression of an endoplasmic reticulum stress-inducing protein (the cog mutant of thyroglobulin), produces a metaphyseal dysplasia phenotype.[25] Consequently, it is likely that abnormal chondrocyte differentiation as a result of the unfolded protein stress response is the main mechanism responsible for the metaphyseal dysplasia, Schmid type, phenotype. The involvement of the unfolded protein response signaling pathway in the pathology offers new therapeutic possibilities in stimulating mutant protein breakdown (by increased proteasomal or autophagic degradation to reduce mutant protein load) or by attenuating the downstream signaling pathways of the unfolded protein response (which impact chondrocyte terminal differentiation).

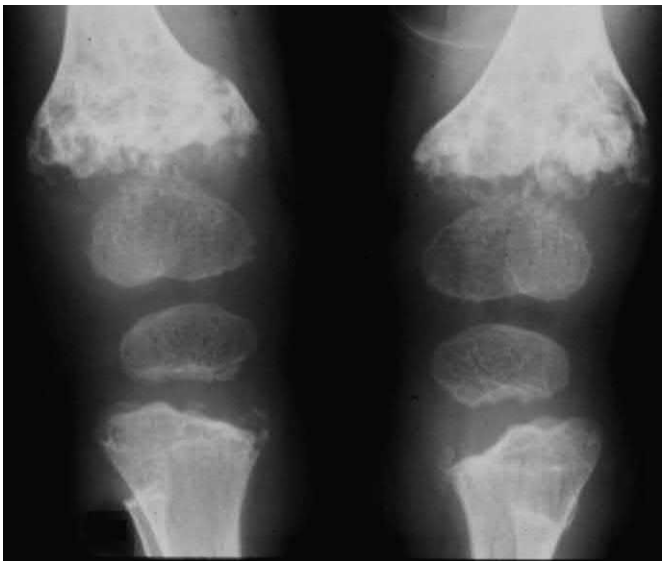

FIGURE 27.5 Radiograph of the knees of a 3-year-old child with AD metaphyseal dysplasia, type Jansen, due to a missense mutation of *PTHR1*. There are extensive changes in the growth plates and metaphyses of the distal femurs and proximal tibias.

The types of metaphyseal dysplasia that present at birth are more severe than the Schmid type and may be associated with other severe phenotypic features. Neonates with metaphyseal dysplasia, Jansen type, are small and have generalized skeletal abnormalities (Fig. 27.5), including brachycephaly, micrognathia, prominent eyes, hypertelorism, choanal stenosis, wide cranial sutures, high-arched palate, short ribs, generalized osteopenia, bowing of the long bones, and markedly expanded cup-shaped metaphyses with features of rickets. Laboratory investigations show hypercalcemia, hypophosphatemia, hypercalcuria, increased urinary excretion of cyclic AMP, elevated 1,25-$(OH)_2$ vitamin D, elevated alkaline phosphatase, and low-to-undetectable levels of parathyroid hormone (PTH) and parathyroid hormone–related protein (PTHrP).

Some patients with AD metaphyseal dysplasia, Jansen type, are heterozygous for activating mutations of the

PTHR1 gene, which encodes the PTH/PTHrP receptor 1. COS-7 cells expressing the mutation showed ligand-independent cAMP accumulation. In a consanguineous Turkish family with the related Eiken syndrome, affected individuals were homozygous for a 1656C>T transition in the last exon of the *PTHR1* gene, resulting in a truncation mutation, arg485 to ter (R485X) in the C-terminal cytoplasmic tail of the protein.[26,27] Radiographs show delayed ossification of the skeleton but, in contrast to metaphyseal dysplasia, Jansen type, the levels of serum calcium, phosphorus, and 1,25-$(OH)_2$ vitamin D were normal and the serum PTH levels were mildly elevated.

While the AD metaphyseal dysplasia, Jansen type, and the AR Eiken syndrome are due to *PTHR1* mutations that delay endochondral bone formation, the AR metaphyseal dysplasia, Blomstrand type, is characterized by advanced endochondral bone formation due to inactivating mutations of *PTHR1*. Mice lacking *Pthr1* die in midgestation, but those that survive show a metaphyseal dysplasia, Blomstrand type, phenotype due to accelerated maturation of the growth plate and accelerated endochondral ossification.

CHH (also known as metaphyseal chondrodysplasia, McKusick type) is another type of metaphyseal dysplasia that is evident at birth. It is an AR form of short-limb dwarfism. The skeletal phenotype consists of flaring of the lower rib cage, lumbar lordosis, occasional mild odontoid hypoplasia, narrowing of interpedicular distances, mild scoliosis, short limbs with mild bowing of the femur, shortening of the tibia relative to the fibula, and widespread irregularities of the metaphyses of the long bones.[28,29] The short hands and feet show joint hyperextensibility. Other features include light-colored, fine and sparse hair, malabsorption, Hirschsprung disease, esophageal atresia, anomalies of the bone marrow and immune system, and an increased risk of malignancy, especially lymphoma and skin tumors. These widespread phenotypic features are due to recessive mutations in the mitochondrial RNA-processing endoribonuclease gene, *RMRP*.

Similar skeletal changes are also present in AR metaphyseal dysplasia without hypotrichosis or immunodeficiency. Some patients have subclinical microscopic evidence of hair hypoplasia. Metaphyseal dysplasia with hypotrichosis results from mutations of *RMRP* and so is allelic to CHH.[30,31]

SBDS is another AR form of metaphyseal dysplasia that manifests at birth. Like CHH, it is associated with a wide range of phenotypic changes beyond the skeleton. Babies are small at birth with failure to thrive. Respiratory distress in the neonatal period is partly related to the short ribs and narrow thorax. The costochondral junctions are thickened with cupping of the anterior metaphyseal ends of the ribs due to metaphyseal dysplasia. There is metaphyseal dysplasia of the long bones and ovoid vertebral bodies. The growth plates are widened and irregular, and the metaphyseal and diaphyseal bones are osteopenic. The extraskeletal manifestations include exocrine pancreatic insufficiency with pancreatic lipomatosis, nephrocalcinosis, developmental delay, bone marrow dysfunction with susceptibility to myelodysplasia and acute myelogenous leukemia, and increased susceptibility to infection. The severity of the manifestations vary considerably and the pancreatic function may improve with age. There is a tendency toward normalization of the epiphyseal maturation defect and progression of the metaphyseal changes with age. Homozygous or compound heterozygous mutations have been identified in the *SBDS* gene that are involved in ribosomal RNA and ribosome biogenesis. The underlying mechanisms that account for the spectrum of phenotypic features are currently unclear. However, one possibility is that the defect in ribosome biogenesis has its greatest impact in cells that have very high levels of protein synthesis, particularly proteins for secretion. Chondrocytes, osteoblasts, pancreatic exocrine cells, and glial cells produce and secrete large amounts of protein in cartilage, bone, pancreas, and nervous system, respectively.

Another form of metaphyseal dysplasia that is often present at birth is metaphyseal anadysplasia, which is a self-correcting skeletal phenotype without extraskeletal manifestations. Neonates have rhizomelic short limbs with radiographic evidence of severe metaphyseal dysplasia and generalized osteopenia. The babies lack the extraskeletal manifestations that occur in the SBDS and CHH and lack the biochemical features of metaphyseal dysplasia, Jansen type. Spontaneous recovery of the skeletal phenotype occurs after 2–3 years. The rhizomelic shortening resolves with the resumption of normal growth (Figs. 27.6–27.8). Although the limbs may be

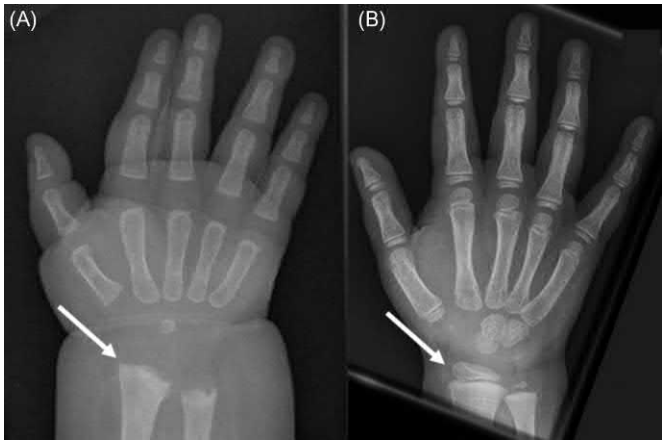

FIGURE 27.6 Radiographs of the hands of a child with AD metaphyseal anadysplasia type 1 due to a missense mutation of *MMP13*. (A) At 3 months of age, there is diffuse osteopenia, as well as severe metaphyseal changes in the distal radius *(arrow)* and ulna. (B) At 6 years of age, the bone density, as well as the growth plate, metaphyseal, and epiphyseal anomalies have spontaneously resolved *(arrow)*.

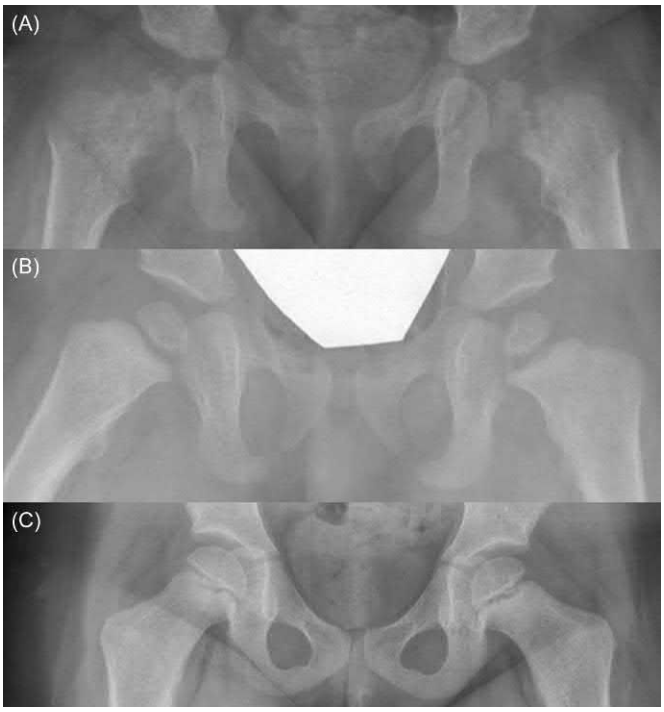

FIGURE 27.7 Radiographs of the hips of a child with AD metaphyseal anadysplasia type 1 due to a missense mutation of *MMP13*. (A) At 4 months of age there is generalized osteopenia. There are extensive abnormalities in the proximal femurs. (B) At 2 years of age, her gait was near normal. Spontaneous improvement was observed in the bone quality and in the development of the proximal femurs and hips. (C) At 6 years of age, her rhizomelic limb shortening and short stature had resolved. She had no clinical abnormalities although her knee radiographs showed some residual widening of the distal femoral growth plates.

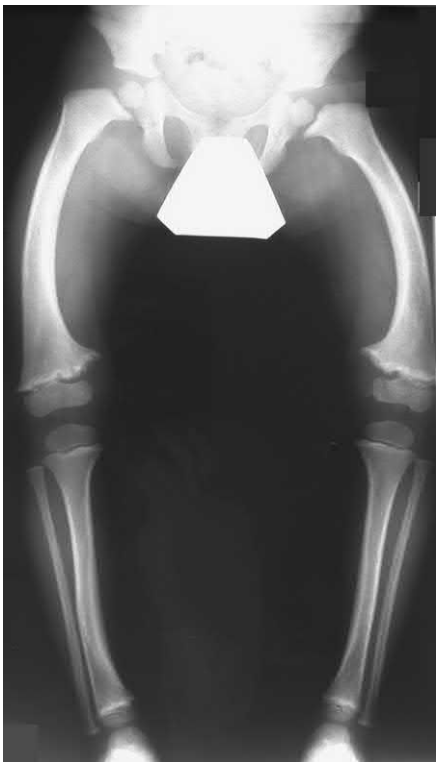

FIGURE 27.8 Radiograph of the legs of a 6-year-old child with metaphyseal dysplasia resembling the Schmid type but without a detectable mutation in *COL10A1*. The child was shown to be heterozygous for a missense mutation in MMP13.

clinically normal, there may be radiographic evidence of residual mild rachitic changes in the growth plates.

There are two forms of metaphyseal anadysplasia, type 1 (MANDP1) and type 2 (MANDP2). Type 1, with either AD or AR inheritance, is caused by mutations in the matrix metalloproteinase 13 gene (*MMP13*). Type 2, which is AR, is caused by mutations in the metalloproteinase 9 gene (*MMP9*). An explanation for the resolving nature of the skeletal phenotype is revealed from studies of *Mmp13*-null mice.[32,33] The *Mmp13* gene is expressed in terminal hypertrophic chondrocytes of the growth plate and in osteoblasts. The severity of the metaphyseal dysplasic phenotype of the growth plates of the *Mmp13*-null mice worsened for about 5 weeks, at which time they spontaneously improved with complete resolution by 12 weeks of age. Further studies showed that degradation of cartilage collagen and aggrecan is a coordinated process in which MMP13 works synergistically with MMP9. Mice lacking both MMP13 and MMP9 have severely impaired endochondral bone formation, characterized by diminished remodeling of the extracellular matrix, prolonged chondrocyte survival, delayed vascular recruitment, and defective formation of trabecular bone.

Metaphyseal anadysplasia type I is allelic to spondyloepimetaphyseal dysplasia, Missouri type.[34,35] The latter dysplasia is an AD disorder also due to mutations of *MMP13*.

6 CONCLUSIONS

The skeletal dysplasias encompass a fascinating array of abnormal bone phenotypes. They also provide novel insights into genes and pathways associated with abnormal skeletal development. The interrelations between phenotype and genotype are complex, variants of a gene may be associated with more than one dysplasia and, conversely, a dysplasia may be associated with variants of more than one gene. Studying these interrelationships can reveal important insights into the function of genes and their effects on skeletal development. With the rapid adoption of next-generation sequencing technologies, the remaining bone dysplasia genes will most likely be discovered within the next few years. A complete list of all genes involved in skeletal dysplasias will be an invaluable asset for studying bone biology in health and

disease. This will not only ultimately lead to better treatments for these rare disorders, but also for more common disorders of bone and cartilage, such as osteoporosis and osteoarthritis.

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CHAPTER

28

Hypophosphatasia and How Alkaline Phosphatase Promotes Mineralization

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1 INTRODUCTION

Hypophosphatasia (HPP) is the inborn error of metabolism and metabolic bone disease that features low serum ALP activity (hypophospha*tasemia*).[1,2] This biochemical hallmark originates from loss-of-function mutation(s) of the gene that encodes the "tissue-*nonspecific*" isoenzyme of ALP (TNSALP)[3,4] expressed ubiquitously and richly in the skeleton, liver, and kidney.[5] In HPP, the genes for the tissue-*specific* ALP isoenzymes expressed separately in the intestine, placenta, and germ cells are presumably intact. Delineation of the clinical, radiographic, biochemical, and histopathological features of HPP and then revelation of its genetic basis verified a role for ALP in bone mineralization first postulated in 1923 by the enzyme's discoverer, Robert Robison, PhD.[6] ALPs are cell-surface phosphohydrolases.[7,8] Thus, TNSALP's natural substrates accumulate extracellularly in HPP—including the inhibitor of hydroxyapatite (HA) crystal growth, inorganic pyrophosphate (PP_i).[9,10] The superabundance of extracellular PP_i (ePPi) explains the rickets or osteomalacia and dental disease that characterizes HPP.[1,2] Now it seems that in other conditions excessive TNSALP contributes importantly to ectopic mineralization.[11,12]

What follows is: (1) a description of the genes for and composition of the ALPs found in humans, (2) a discussion of how the skeleton mineralizes, (3) a summary concerning how TNSALP functions in skeletal and perhaps ectopic mineralization, (4) a review of HPP, and (5) the lessons HPP has taught us about TNSALP, including confirmation of Robison's hypothesis from successful TNSALP replacement therapy for HPP.

2 MOLECULAR BIOLOGY AND BIOCHEMISTRY OF ALKALINE PHOSPHATASE

ALP (orthophosphoric-monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1) is found in all animals.[13] In humans, ALPs derive from four genes.[5,14] Three genes each encode a single tissue-specific ALP isoenzyme; that is, "intestinal," "placental," or "germ-cell (placental-like)" ALP. The fourth gene encodes an ALP that is expressed ubiquitously and richly in the skeleton, liver, and kidney.[5,14] Accordingly, this ALP isoenzyme is called TNSALP. Clinicians have recognized for many years[13] that the ALP found in bone and liver differs. However, the dissimilarity is lost following digestion with glycosidases because "TNSALP" actually comprises a family of "secondary" isoenzymes (isoforms) differing only by posttranslational modifications involving carbohydrates.[5,14]

The TNSALP gene (OMIM *171760)[15] is near the end of the short arm of chromosome 1 (1p36.1-34),[16] whereas the genes for intestinal, placental, germ-cell, and perhaps a fetal intestinal ALP (OMIM *171740, *171800, *171810, *171750, respectively)[15] are near the end of the long arm of chromosome 2 (2q34-37).[17] The human gene mapping symbol for the TNSALP locus is ALPL (ALP-liver), yet the role for bone not liver TNSALP is understood (see later). Accordingly, over the years, I have used *TNSALP* not *ALPL* as the better designation for the gene.[1,2]

Each ALP gene in humans was sequenced in the 1980s.[18–20] *TNSALP* seems to be the ancestral gene from which the tissue-specific ALPs arose by gene duplication.

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TNSALP extends more than 50 kb and has 12 exons. Eleven exons are translated to the 507 amino acid nascent enzyme.[14,20] The 5′-untranslated region differs between the bone and liver TNSALP isoforms.[21] The promoter region is 610 nucleotides 5′ to the major transcription start site,[22] and TATA and Sp1 sequences seem important for its function. Basal expression of TNSALP appears to reflect "housekeeping" promoter activity, whereas enhanced levels in certain tissues may be mediated by a posttranscriptional mechanism.[22]

The tissue-specific ALP genes are smaller than *TNSALP*, primarily due to shorter introns.[18–20] Amino acid profiles deduced from their cDNAs suggest 87% positional identity between placental and intestinal ALP, but only 50%–60% identity between them and TNSALP.[14] However, the active site of these ALPs is encoded by six exons using base sequences conserved throughout nature.[23]

Human ALP gene sequence indicates that the nascent enzyme has a short signal peptide of 17–21 amino acid residues and a hydrophobic domain at its carboxy-terminus.[18–20] Actually, ALPs tether to the external surface of the plasma membrane by binding to the polar head group of a phosphatidylinositol-glycan moiety. The precise interaction may, however, differ among the ALP isoenzymes.[14]

The cDNA sequence of TNSALP predicts five potential N-linked glycosylation sites,[20] a modification necessary for catalytic activity.[21] O-glycosylation involves the bone, but not the liver, isoform.[21]

In tissues, ALPs function as homodimers and possibly as homotetramers.[23] Catalytic activity requires a multimeric configuration of identical subunits with molecular mass ranging from 40 to 75 kDa.[14,24] TNSALP in symmetric dimeric form has α/β topology for each subunit with a 10-stranded β-sheet at its center.[14] Each monomer has one active site and two Zn^{2+} atoms that stabilize its tertiary structure.[13,25]

In vitro the ALPs have broad substrate specificity and pH optima conditioned by the type and concentration of phosphocompound undergoing catalysis.[13] Hydrolytic activity cleaves phosphoesters and PP_i.[26] Mg^{2+} is an essential cofactor.[13] The process involves phosphorylation-dephosphorylation of a serine residue, and dissociation of covalently linked phosphate seems to be the rate limiting step for catalysis. Inorganic phosphate (P_i) is a potent competitive inhibitor of ALP,[13,25] but may stabilize the enzyme.[27] Recent evidence indicates that TNSALP can also dephosphorylate the polyphosphorylated Sibling protein regulators of skeletal mineralization including osteopontin (see later).[28]

In health, circulating ALPs are generally lipid-free homodimers.[13] In adults, blood contains approximately equal amounts of TNSALP from bone and liver (hepatobiliary) tissue.[29] In infants and children, and especially during the growth spurt of adolescence, the circulation is particularly enriched with the bone isoform of TNSALP.[13] Placental ALP biosynthesis is controlled by the fetal genome, and in health this isoenzyme is detected in serum only from pregnant women during the last trimester.[13,14] Various malignancies express and can release placental or placental-like (germ cell) ALP into the bloodstream.[14] Some people (with B and O blood types and positive secretory status) increase after a fatty meal the typically small amount of circulating intestinal ALP.[13,14,30]

How ALPs are released in vivo from cell surfaces is poorly understood. In vitro, ALPs can be liberated by phosphatidylinositol-specific phospholipase.[14,24] Clearance of circulating ALP presumably occurs, as for other glycoproteins, in the liver.[31]

3 PHYSIOLOGY OF SKELETAL MINERALIZATION

Skeletal development is complex and involves bone growth, modeling (shaping), and remodeling (formation and resorption, or "turnover"). Elongation of long bones in the extremities and bone growth at various sites in the axial skeleton occurs by an endochondral process that largely finishes after puberty. In healthy physes (growth plates), orderly proliferation, hypertrophy, and then degeneration of chondrocytes precedes mineralization and vascularization of the elaborated cartilage matrix. Following resorption of this calcified "primary spongiosa" by osteoclasts (OCs), osteoblasts (OBs) synthesize bone matrix (osteoid), which then mineralizes as "secondary spongiosa."

In 1923, Robert Robison, PhD, discovered that calcifying cartilage and bone from young rats and rabbits was rich in phosphatase activity.[6] He hypothesized that the enzyme conditioned skeletal mineralization by increasing the local concentration of P_i by hydrolyzing possibly a hexosephosphoric ester.[6] One year later, Robison and Soames reported that this phosphatase in vitro had a significantly alkaline pH optimum.[32] However, Robison realized that at such high pH and using colorometric substrates his assay was not physiological and never used the term "ALP." Instead, he referred to the enzyme as "bone phosphatase."[33] Robison later realized that a further, but unknown, factor other than P_i release by bone phosphatase conditioned skeletal mineralization.[33] The term "ALP" emerged in the 1930s when bone specimens from different skeletal disorders were found to have phosphatase activity with either acid or alkaline pH optima.[33a] Soon after, measuring phosphatase activity in serum in the clinical laboratory using colorimetric substrates at high pH was found useful by clinicians to detect and to follow the course of skeletal as well as hepatobiliary disease.[13] Now we know that although

the hydrolytic rates might be reduced the pH optima of ALPs are less alkaline for natural substrates at physiological concentrations.[5,13,14] Nevertheless, nearly a century after the discovery of ALP, the methods used to assay this enzyme's activity do not reflect its physiological function (see later).[3,4] In clinical and research laboratories both, ALP activity is still measured in artificial buffers containing high (mM) concentrations of colorimetric substrates (e.g., *p*-nitrophenylphosphate) at nonphysiological alkaline pH (e.g., 9.2–10.5).[13]

Soon after its discovery, ALP was found abundant also in tissues that do not mineralize (e.g., liver, intestine, placenta).[13] Thus, its role in skeletal mineralization was challenged. Instead, the physiological function was postulated to include hydrolysis of phosphate esters to supply the nonphosphate moiety, synthesis of phosphate esters with ALP functioning as a transferase, or regulation of cellular processes in which ALP acted as a phosphoprotein phosphatase.[34] Indeed, relatively many hypotheses were offered early on for how TNSALP could function in skeletal mineralization.[34] These included as: a plasma membrane transport protein for P_i, an extracellular calcium (Ca) binder that promotes Ca–P_i formation and orients HA crystal deposition into osteoid,[35] a Ca^{2+}/Mg^{2+}-ATPase, or, a phosphoprotein phosphatase that prepares skeletal matrix for ossification.[36] To fulfill Robison's hypothesis,[33] the P_i donor was postulated instead to be ATP from degenerating cells.[37] Robison's unknown second factor for regulating skeletal mineralization[33] was revealed in 1966 when Fleisch et al.[38] reported that PP_i is an endogenous inhibitor of mineralization by impairing HA crystal growth. The endogenous levels of PP_i were then found to be increased in HPP,[9,10] thus explaining the disorder's skeletal and dental complications.[39] ALP was subsequently shown to be a PP_i-ase.[14,40]

Hypertrophic chondrocytes and OBs are rich in bone TNSALP. In the late 1960s, H. Clark Anderson, MD using electron microscopy discovered that the earliest site of HA deposition during endochondral bone formation occurs near hypertrophic chondrocytes within membrane-bound structures called matrix vesicles (MVs).[41] MVs are presumed to be buds of the plasma membrane of chondrocytes and OBs, and appear also in growing membranous bone and fracture callus.[41] MVs are rich in pyrophosphatase (PP_i-ase) and ATPase activity and may contain polysaccharides, phospholipids, and glycolipids.[41] HA crystals grow within and eventually rupture MVs, and then enlarge extravesicularly before being deposited into osteoid.[42] Accordingly, skeletal mineralization can be considered "primary" or "phase 1" occurring in MVs, and then "secondary" or "phase 2" featuring extravesicular HA crystal enlargement within the skeletal matrix.[43]

In ectopic mineralization (e.g., vascular calcification of chronic renal failure, diabetes mellitus), the soft tissue calcification may be from various perturbations, such as inflammation that increase ALP activity locally and thereby decrease ePPi levels.[11,12]

Generalized impairment of skeletal (i.e., cartilage and bone) matrix mineralization in infants, children, or adolescents compromises endochondral and intramembranous bone formation and leads to the constellation of clinical abnormalities referred to as "rickets." When such impaired mineralization begins in an adult (i.e., after growth plate fusion) the consequences are "osteomalacia." The principal feature that distinguishes rickets from osteomalacia is disruption of physeal and apophyseal function that typically causes short stature and skeletal deformity. Nearly all forms of rickets or osteomalacia derive from low extracellular levels of Ca and/or P_i. Elevated ALP activity in the circulation (i.e., "hyperphosphatasemia," "hyperphosphatasia") is expected by clinicians and would seem to reflect "priming" occurring in hard tissues to foster any potential mineralization. HPP is therefore an extraordinary and seemingly paradoxical type of rickets/osteomalacia.

4 HYPOPHOSPHATASIA

The discovery of HPP in 1948,[44] its further clinical and laboratory delineation, and ultimately proof of its etiology 40 years later[3] verified Robison's hypothesis[6] and provided our greatest insight into the physiological role of ALP.[1,2,5] With confirmation beginning in 1988[3] that HPP is, as would be predicted, caused by loss-of-function mutation(s) within the gene encoding TNSALP, unequivocal evidence united ALP with mineralization of the bones and teeth. Additionally, a key role for TNSALP in vitamin B_6 metabolism was identified (see later). Muscle weakness in HPP at first seemed enigmatic.[1,2] However, undisturbed function of other organs/tissues in HPP, even TNSALP-rich liver and kidney, argues against further biological significance for TNSALP.[3,5] In severe HPP (see later), dysfunction elsewhere can be a direct or indirect consequence of the global impairment of mineralization.[1,2]

4.1 History

In 1948, a young Canadian pediatrician, John C. Rathbun, MD, coined the term "HPP" when he published his report of an infant boy born in 1946 who acquired and then died at 2 months-of-age from severe rickets and seizures yet whose ALP activity in serum (and in bone and other tissues obtained at autopsy) was paradoxically subnormal.[44] This was the discovery of HPP. Some 50 years after Rathbun's publication, we obtained DNA from both parents that indicated their baby was compound heterozygous for different

missense mutations in *TNSALP*.[45] There are earlier case reports that probably represent encounters with this disorder.[46,47] Then, in 1953, premature loss of deciduous teeth was identified as the second major clinical feature of HPP (see later).[48] Subsequently, muscle weakness,[49] vitamin B_6-dependent seizures,[50] and craniosynostosis[51] were recognized to be important potential complications when HPP is severe.[1,2]

The discoveries of elevated endogenous levels of three phosphocompounds in HPP clarified its metabolic basis and the physiological role of TNSALP.[1,2,5] In 1955, increased levels of phosphoethanolamine (PEA) in urine[52,53] provided a second biochemical marker for HPP. In 1965 and 1971, high levels of PP_i were documented in the urine[9] and blood,[10] respectively, of HPP patients. This revealed why hard tissues failed to mineralize properly (see later). In 1985, elevated plasma levels of pyridoxal 5′-phosphate (PLP), the major circulating form of vitamin B_6, were discovered in HPP and indicated that TNSALP functions as a cell surface enzyme (see later).[7] In 1988, when the structure of the TNSALP gene was characterized by Weiss et al.,[20] a homozygous loss-of-function mutation of *TNSALP* was identified in another Canadian infant boy who died from HPP.[3] This began what is my expectation that a *TNSALP* mutation(s) underlies all *bona fide* HPP patients.[54] In 2015, bone-targeted recombinant TNSALP-replacement therapy (asfotase alfa) was approved by regulatory agencies multinationally to treat typically pediatric-onset HPP (see later).[54]

4.2 Clinical Features

More than several hundred HPP patients are now mentioned in the medical literature and the clinical, radiological, biochemical, skeletal histopathological, and genetic features of HPP, as well as its extraordinarily broad ranging severity, are established (see later).[1,2] The life-threatening severe forms of HPP presenting in neonates and babies are now known to reflect autosomal recessive (AR) inheritance, whereas relatively mild HPP typically reflects autosomal dominant (AD) inheritance of TNSALP mutations with dominant/negative effects (see later).[55]

HPP is most prevalent among Mennonites in Manitoba, Canada, where ~1 in 2500 newborns manifests lethal HPP,[56] and ~1 in 25 individuals carries their *TNSALP* founder mutation.[57] In 1957, the incidence of severe HPP in Toronto, Canada, was estimated to be 1 per 100,000 live births.[47] In 2011, based upon *TNSALP* mutation data, the prevalence of "severe" and "moderately severe" HPP was estimated in France to be 1/300,000 and 1/6,370 individuals, respectively.[58] HPP seems to be a disease primarily of Caucasians,[55] but has been reported in Japan,[59–62] and occurs in Hispanic, Native-American, and Chinese people although the prevalences are not known. Inexplicably, HPP seems to be particularly rare with black ancestry.[63]

Despite relatively high levels of TNSALP in healthy people not only in bone but also in liver, kidney, and adrenal tissue (and some TNSALP throughout the body), HPP when severe directly disrupts hard tissues (skeleton and teeth). The muscle weakness too may reflect toxicity from elevated ePPi levels,[1,2] whereas the vitamin B_6-dependent seizures are instead from impaired PLP hydrolysis[1,2,50] (see later). However, the expressivity of HPP ranges remarkably from death in utero from an unmineralized skeleton to problems only with dentition or calcific arthropathy in adult life.[47,64–68] I regard HPP as having the broadest expressivity of all skeletal diseases.[55] Although HPP generally "breeds true" in siblings, significantly different disease severity can occur despite an identical *TNSALP* genotype[65,66,69,70] because other factors beyond the underlying mutation(s) may be at issue.[1,2] Additionally, some individuals who demonstrate hypophosphatasemia and TNSALP substrate accumulation characteristic of HPP and harbor a defective *TNSALP* allele I consider "carriers" who perhaps will never become symptomatic.[55]

Several clinical classification schemes have been proposed for HPP that attempt to deal with its remarkable range of expressivity.[47,64] The extant nosology for HPP was initiated by Douglas Fraser, MD in 1957.[47] Seven principal clinical forms, compiled later, represent a useful classification of HPP. However, it is clear that HPP is in reality a broad-ranging disease continuum or spectrum.[1,2]

HPP patients who manifest only dental complications without skeletal disease have odonto-HPP, likely the most prevalent HPP.[55] If HPP bone disease or other important HPP problems are present, the age when they became apparent distinguishes the perinatal, infantile, childhood, and adult forms.[47,55] Now, childhood HPP can be distinguished as "mild" or "severe".[55] Further types include benign prenatal HPP that causes skeletal deformity in utero or at birth, but in contradistinction to perinatal HPP (see later) is clearly more mild and shows significant spontaneous postnatal improvement.[71] The rarest HPP variant, pseudo-HPP,[72,73] resembles infantile HPP except that serum ALP activity is normal or high in the nonphysiological assay of the clinical laboratory (see later).

The prognosis for the various forms of HPP is largely conditioned by the severity of the skeletal disease which, in turn, reflects the age at presentation and diagnosis.[1,2] Usually, the earlier HPP bone disease becomes symptomatic the worse the outcome.[3,37] The remarkable exception is benign prenatal HPP that is apparent at birth but then improves spontaneously with an aftermath that ranges from infantile HPP to odonto-HPP (see later).[71]

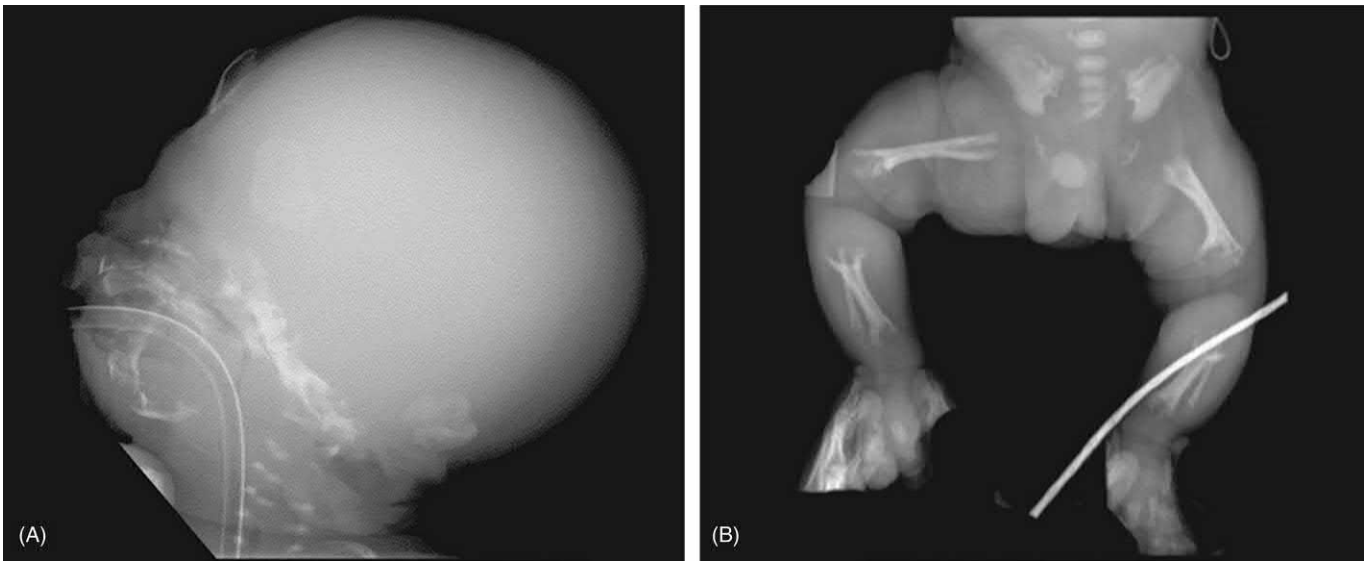

FIGURE 28.1 Perinatal hypophosphatasia. (A) Lateral radiograph of the skull of this newborn shows extreme hypomineralization. (B) Marked rachitic changes are seen in the long bones of the lower extremities. *Source: Reproduced with permission from Whyte MP.* Hypophosphatasia, in "pediatric bone: biology & diseases" *3rd ed. In: Glorieux FH, Jueppner H, Pettifor J, editors. San Diego (CA): Elsevier (Academic Press); 2012. p. 775.*

Although the above nosology for HPP is useful,[54] there is considerable overlap for its signs, symptoms, complications, and prognosis between the individual clinical groupings,[55,74] and separation into several forms remains artificial. As described below, I believe that HPP spans the greatest range of severity among all skeletal diseases.

4.2.1 *Perinatal Hypophosphatasia*

This is the most severe form of HPP, and unless treated promptly with asfotase alfa (see later) is nearly always fatal.[56,75] It manifests in utero and can cause stillbirth. The pregnancy may be complicated by polyhydramnios. At delivery, limbs are short and deformed and there is *caput membraneceum* from profound skeletal hypomineralization including the skull. Unusual osteochondral (Bowdler) spurs may extend laterally from the midshaft of the ulnas and fibulas and pierce the skin.[76–78] Some newborns have a high-pitched cry, irritability, periodic apnea with cyanosis and bradycardia, unexplained fever, myelophthisic anemia (perhaps from marrow space crowding by excess osteoid and unmineralized cartilage), or intracranial hemorrhage.[64,67] They live a few days but suffer increasing respiratory compromise from defects in the thorax and perhaps hypoplastic lungs.[79] Very rarely does intensive supportive care alone bring long-term survival.[80,81]

Radiographs of the skeleton show pathognomonic findings,[76,77] distinguishable from and more severe than even the worst instances of osteogenesis imperfecta or other congenital dwarfism. Nevertheless, the features can be diverse, and with patient-to-patient variation.[77] Sometimes, bones appear completely unmineralized on radiographic examination (Fig. 28.1A). Parts of (or entire) vertebrae may seem missing. If some skeletal mineralization is present, profound rachitic changes are apparent. The findings can include poorly ossified epiphyses together with irregular extensions of radiolucency into metaphyses (Fig. 28.1B). Fractures are often present. Individual membranous bones of the cranium may show calcification only centrally, giving the illusion that the cranial sutures are wide, yet they are functionally closed.[76] The teeth are poorly formed.[77]

4.2.2 *Infantile Hypophosphatasia*

This form of HPP presents postnatally, but before 6 months-of-age.[47,80] Development may seem normal until the onset of poor feeding, inadequate weight gain, failure-to-thrive, hypotonia, clinical signs of rickets, or seizures.[80] The cranial sutures feel wide, but this is explained by diminished ossification of the skull. There may be bulging of the anterior fontanel, raised intracranial pressure with papilledema, proptosis, mild hypertelorism, and brachycephaly. Sclerae can be blue. A flail chest that predisposes to pneumonia may occur from rachitic deformity of the thorax and rib fractures.[75] Weakness and delayed motor milestones are important complications.[80] Rarely, pyridoxine-dependent seizures occur before skeletal disease becomes apparent, and a lethal outcome from subsequent rickets is predicted.[50] Hypercalcemia and hypercalciuria from blocked mineral entry into the skeleton is common, and can cause recurrent vomiting and nephrocalcinosis with renal compromise, respectively.[47,82] If the baby survives infancy, true bony fusion of the cranial sutures may occur prematurely and cause neurological sequelae.[51,76]

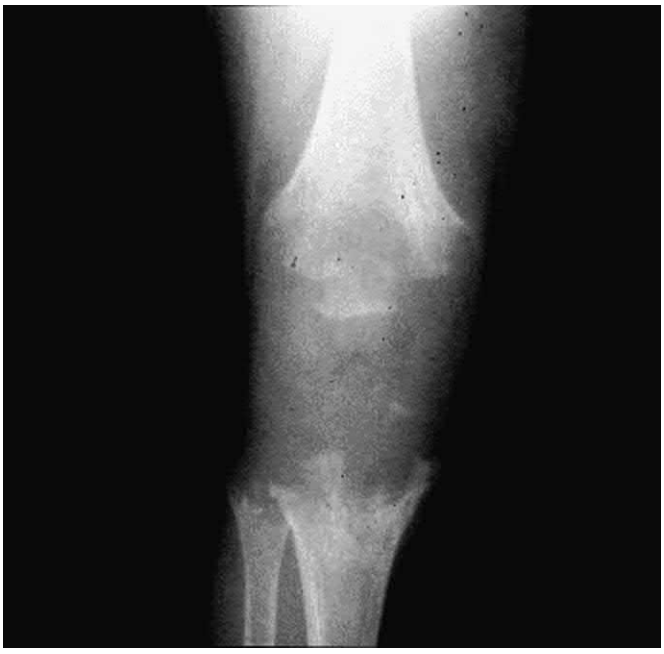

FIGURE 28.2 Infantile hypophosphatasia. Anteroposterior radiograph of the right knee of this 10-month-old boy shows pathognomonic changes of severe hypophosphatasia including extremely irregular metaphyses with tongues of radiolucency and areas of osteosclerosis. *Source: Reproduced with permission from Whyte MP.* Hypophosphatasia, in "pediatric bone: biology & diseases" *3rd ed. In: Glorieux FH, Jueppner H, Pettifor J, editors. San Diego (CA): Elsevier (Academic Press); 2012. p. 775.*

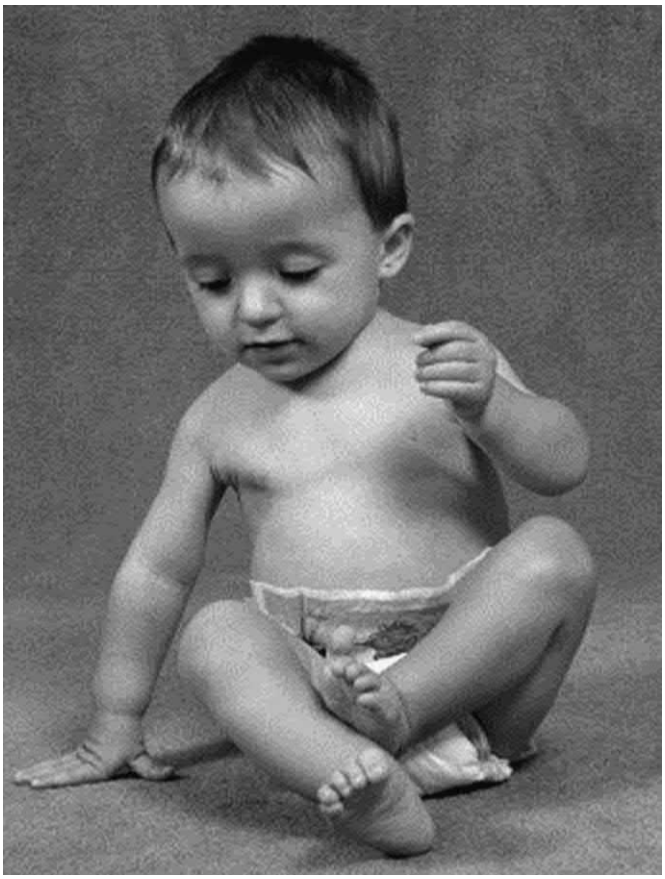

FIGURE 28.3 Infantile hypophosphatasia. This 19-month-old boy is a survivor of infantile hypophosphatasia. Note his broad forehead and flared wrists and ankles from rachitic widening of metaphyses.

Although somewhat less severe than in the perinatal form of HPP, the radiographic changes of infantile HPP are also remarkable and pathognomonic (Fig. 28.2).[76,80] Sometimes there is an abrupt transition from normal appearing diaphyses to poorly calcified metaphyses suggesting a sudden worsening of mineralization during endochondral bone formation.[47] Subsequent radiographic studies may then disclose that this skeletal hypomineralization (rickets) persists, but also show gradual and generalized demineralization of the bones.[80,82] Fractures and progressive deformities accompany such deterioration. Skeletal scintigraphy in infantile HPP can suggest functional closure of cranial sutures if they appear "widened" radiographically and show decreased radioisotope uptake.[83] So-called functional craniosynostosis can occur despite widely "open" fontanels that are instead an illusion from hypomineralized areas of calvarium.

4.2.3 Childhood Hypophosphatasia

This form of HPP is diagnosed after 6 months-of-age, but before adulthood (Fig. 28.3), and itself has a considerable range of severity.[47,84,85] Our investigation reported in 2015 of 173 children with HPP furthermore validated designation of a "severe" and a "mild" form of childhood HPP helping to better organize the nosology for prognostication and further study.[55] In 2016, we published a natural history study of the 101 of these children with longitudinal data.[74]

Premature loss of deciduous teeth (i.e., earlier than the fifth birthday) occurs without tooth root resorption, and instead because of poor mineralization of the cementum that covers and anchors the tooth root.[86] "Baby" teeth slide out from their socket painlessly, without bleeding, and strikingly with their root intact (Fig. 28.4). The lower and then upper incisors are typically lost first. The mean number of teeth lost prematurely is 4.2, 6.1, 6.8, and 9.0 for odonto, mild childhood, severe childhood, and survivors of infantile HPP, respectively.[55] Dental radiography may show enlarged pulp chambers and root canals (shell teeth). Alveolar bone attrition, especially in the anterior mandible, can result from the lack of mechanical stimulation.[87] Any cementum itself may be destroyed by periodontal infection.[87] Rachitic deformities can include beading of the costochondral junctions, bowed legs or knock-knees, enlargement of the wrists, knees, and ankles from widened metaphyses, and occasionally a brachycephalic skull (Fig. 28.3).[51] Severe childhood HPP is associated with muscle weakness and often delayed walking and a waddling gait.[1,54] Short stature is,

FIGURE 28.4 Childhood hypophosphatasia. This maxillary central incisor, shown in a "lingual" (posterior) view, was lost spontaneously at 2.5 years of age. Note that the entire root is present—a characteristic of pediatric hypophosphatasia.

however, uncommon.[55] Patients may complain of skeletal pain and stiffness, as well as episodes of joint discomfort and swelling.[84] Rarely, a painful syndrome involving bone marrow edema occurs that mimics chronic recurrent multifocal osteomyelitis or malignancy.[88] However, childhood HPP is not associated with vitamin B_6-dependent seizures.

Radiographs of the major long bones in childhood HPP usually reveal characteristic focal defects of cartilage that project from the growth plates into the metaphyses (Fig. 28.5). These are often described as "tongues" of radiolucency. This feature, if present, can distinguish HPP from other forms of rickets and the metaphyseal dysplasias.[34,76,80,84] There can also be irregularity of the provisional zone of calcification, metaphyseal flaring with areas of radiolucency adjacent to areas of osteosclerosis, and sometimes physeal widening. Secondary centers of ossification (epiphyses) may appear well preserved. Premature bony fusion of all cranial sutures (craniosynostosis) can cause raised intracranial pressure, proptosis, and cerebral damage.[51] Then, the calvarium has a diffuse "beaten-copper" appearance (Fig. 28.6).

Although the prognosis for the permanent dentition is good early on,[89] poorly characterized problems seem

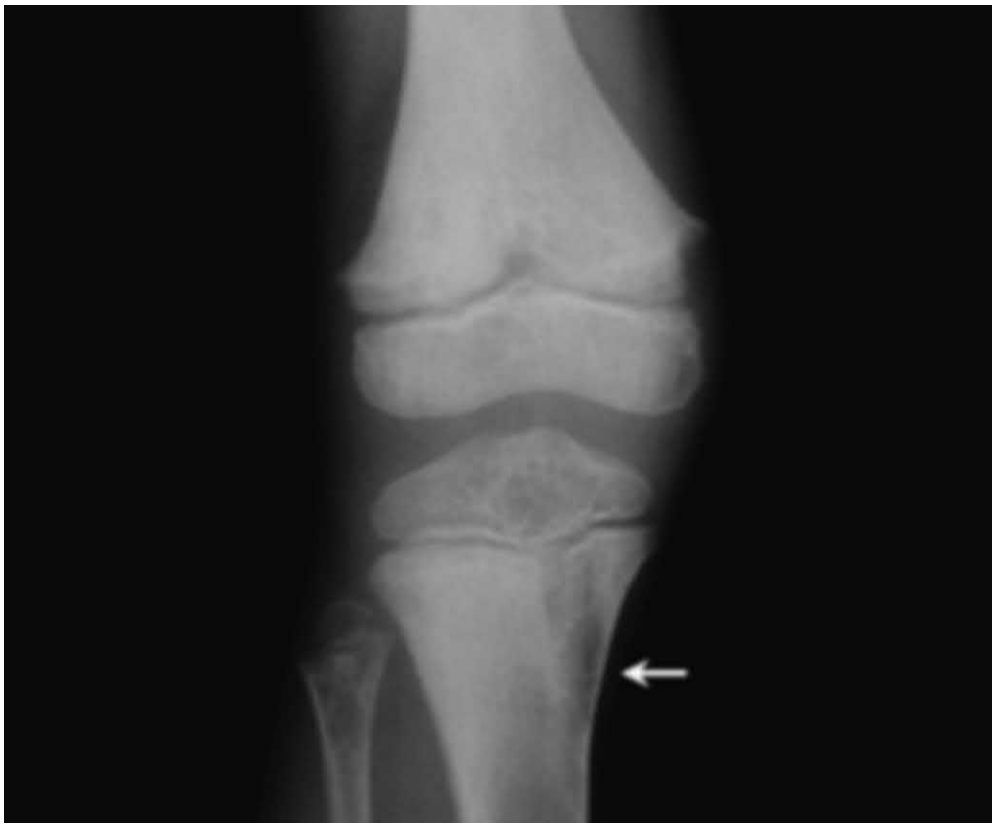

FIGURE 28.5 Childhood hypophosphatasia. Anteroposterior radiograph of the right knee of this 6-year-old boy shows a characteristic "tongue" of radiolucency (arrow) projecting from the physis into the metaphysis where there is paradoxical osteosclerosis. The head of the fibula is particularly involved. *Source: Reproduced with permission from Whyte MP.* Hypophosphatasia, in "pediatric bone: biology & diseases" *3rd ed. In: Glorieux FH, Jueppner H, Pettifor J, editors. San Diego (CA): Elsevier (Academic Press); 2012. p. 776.*

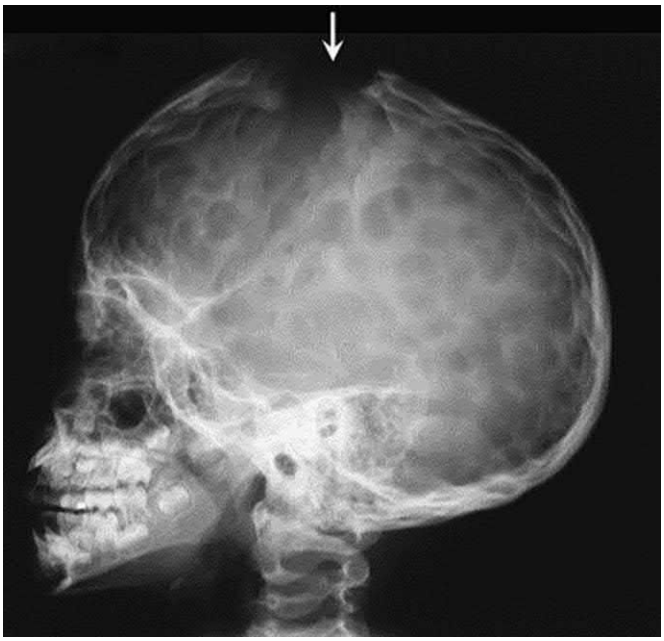

FIGURE 28.6 Childhood hypophosphatasia. Lateral radiograph of the skull of this 5-year-old girl shows pansuture closure causing a "beaten copper" appearance. A craniectomy defect (arrow) is present. *Source: Reproduced with permission from Whyte MP.* Hypophosphatasia, in "pediatric bone: biology & diseases" *3rd ed. In: Glorieux FH, Jueppner H, Pettifor J, editors. San Diego (CA): Elsevier (Academic Press); 2012. p. 777.*

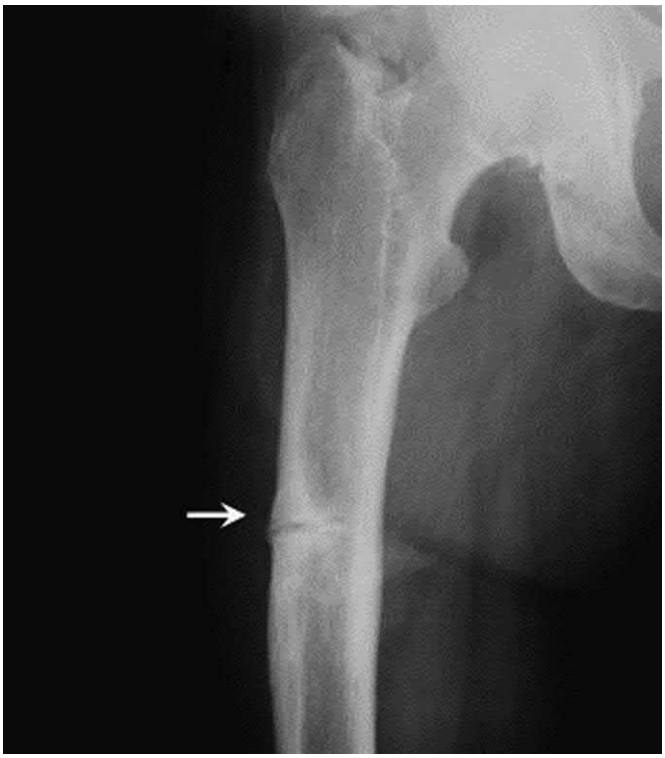

FIGURE 28.7 Adult hypophosphatasia. Anteroposterior radiograph of the proximal right femur of this 51-year-old woman shows a subtrochanteric "stress" fracture in the lateral diaphyseal cortex (*arrow*). Actually, this is a pseudofracture (Looser's zone, Milkman fracture) characteristic of the osteomalacia of hypophosphatasia. *Source: Reproduced with permission from Whyte MP.* Hypophosphatasia, in "pediatric bone: biology & diseases" *3rd ed. In: Glorieux FH, Jueppner H, Pettifor J, editors. San Diego (CA): Elsevier (Academic Press); 2012. p. 777.*

often to lead to tooth loss and denture wearing in adult life (personal observation). Some patients describe greater strength and less pain after puberty, occasionally with radiographic improvement, but the likelihood for eventually developing complications from osteomalacia, and so on, is not understood.

4.2.4 *Adult Hypophosphatasia*

This form of HPP usually presents during middle age.[65,66] Not infrequently, however, patients recount a history of rickets and sometimes premature loss of deciduous teeth. Then, following good health in early adult life, they manifest painful feet caused by recurrent, poorly-healing, metatarsal stress fractures.[90] Subsequently, they can have discomfort in the hips or thighs due to femoral subtrochanteric pseudofractures (see later).[91–93] Polyostotic and nonhealing fractures and pseudofractures may then cause significant morbidity.[94,95] Early loss or extraction of the adult dentition has been commonly reported.[65,66] Calcium pyrophosphate dihydrate (CPPD) deposition in some patients causes PP_i arthropathy,[66] occasionally including attacks of pseudogout. This reflects increased endogenous levels of PP_i (see later).[66,96] There may also be seemingly paradoxical deposition of HA crystals causing calcific periarthritis and ossification of ligaments (syndesmophytes) resembling spinal hyperostosis (Forrestier disease).[68,96,97] Now gaining attention is that these rheumatological features may be common in adults with previously ignored persistent hypophosphatasemia and can be found to harbor a *TNSALP* mutation.[98] Rare instances of primary hyperparathyroidism have been reported.[99]

In adult HPP, radiographs often show pseudofractures (Looser's zones, Milkman fractures),[91–93]—a hallmark of osteomalacia (Fig. 28.7). In the femurs, they characteristically occur proximally and in the lateral cortex rather than medially or in the femoral neck as in other types of osteomalacia.[91] Hence, they resemble the "stress" fractures that are the prodromal lesions for atypical subtrochanteric femoral fractures associated with antiresorptive therapy for osteoporosis.[92] Radiographs may also reveal generalized osteopenia, chondrocalcinosis, features of PP_i arthropathy, and calcific periarthritis.[66,68,96,97]

Sometimes, adult HPP becomes debilitating.[90,100–111]

4.2.5 *Odontohypophosphatasia*

This mildest and likely most prevalent form of HPP is diagnosed when the only apparent complication is its dental disease. Otherwise, health is good.[1,54,74] Here, there is no radiographic or bone biopsy evidence of HPP

skeletal disease.[65] Odonto-HPP may explain some cases of "early-onset periodontitis," although hereditary leukocyte abnormalities and other disorders are usually the cause (see later).

4.2.6 *Pseudohypophosphatasia*

This form of HPP is especially intriguing but exceptionally rare. It features normal or enhanced TNSALP activity in the artificial conditions of laboratory assays,[103] but diminished catalytic activity toward its natural substrates, which therefore accumulate endogenously. Pseudo-HPP has been documented unequivocally but only once.[72,73,101,102] Here, the clinical, radiographic, and biochemical[102] findings are those of infantile HPP except for the serum ALP activity that is consistently normal or elevated when assayed in the clinical laboratory.[72] The heterozygous *TNSALP* mutations are known for the index patient.[73]

Other reports of pseudo-HPP can be unconvincing,[104,105] and probably describe HPP patients with transient normalization of serum ALP activity during fracture, illness, and so on, or more likely misinterpretation of reference ranges for serum ALP activity and/or overemphasis on the significance of a slightly elevated serum or urine PEA level (see later).

4.2.7 *Benign Prenatal Hypophosphatasia*

Beginning in the 1990s, several reports emphasized that some newborns with HPP can manifest bowing deformity in utero or at birth suggestive of perinatal HPP, but instead have postnatal courses featuring spontaneous improvement.[71] Now, this not uncommon form of HPP is well documented and is sometimes referred to as "bent but not broken" HPP. In 2008, it became apparent that AR, as well as AD inheritance of a variety of *TNSALP* mutations can manifest clinically this way (see later).[55,71,106] In 2011, our detailed assessment of 17 such patients together with a review of the literature revealed that the mothers typically carried a defective *TNSALP* allele and that the skeletal deformity of the fetuses tended to be relatively mild and could improve during later stages of pregnancy.[71] However, HPP outcome, when skeletal deformity is detected early in utero[112] by ultrasound, is not predictable.[71]

4.3 Laboratory Findings

4.3.1 *Biochemical*

4.3.1.1 ALP Activity

HPP can be diagnosed confidently when the clinical history, physical and biochemical findings, and radiographic changes match with serum ALP activity that is clearly subnormal for the patient's age.[1,2,54] In general, the more severe the HPP the more severe the hypophosphatasemia.[2] Even patients with odonto-HPP are consistently hypophosphatasemic. In perinatal and infantile HPP, low serum ALP activity is detectable at birth using umbilical cord blood.[106] Notably, in forms of rickets or osteomalacia other than HPP, serum ALP activity is typically increased. Hence, the hypophosphatasemia of HPP seems paradoxical and is especially striking. Nevertheless, several diagnostic pitfalls must be avoided (Table 28.1). Blood for serum ALP assay must be collected properly.[2,90,100] Chelation of Mg^{2+} or Zn^{2+} by ethylenediamine tetra-acetic acid (EDTA) will destroy ALP activity.[13] Furthermore, levels of serum ALP activity should be interpreted knowing that reference ranges differ significantly according to patient age and gender.[13] Healthy infants, children, and adolescents have considerably higher-serum ALP levels compared to adults (reflecting more bone TNSALP). Also, the peak serum ALP activity of the growth spurt of adolescence occurs earlier in girls than in boys.[13] Although the problem is waning, here in 2017 reference ranges cited by some clinical laboratories are for adults exclusively, and sometimes with no lower limit (personal observation). This is likely because clinicians typically concern themselves with elevated circulating ALP values to detect and to follow other skeletal or hepatobiliary diseases.[13] Consequently, for some infants or children the diagnosis of HPP is missed because they are erroneously considered to have normal serum ALP levels, or are mistakenly diagnosed with pseudo-HPP. Furthermore, hypophosphatasemia may occur in a variety of other disorders, exposure to certain drugs (glucocorticoids, chemotherapy, clofibrate, vitamin D toxicity, or milk-alkali syndrome), massive transfusion of blood or plasma, or radioactive heavy metal poisoning (Table 28.1).[2,113] However, these situations should be readily apparent and diagnosed.

TABLE 28.1 Causes of Low Serum Alkaline Phosphatase (ALP) Activity (Hypophosphatasemia)

Cardiac bypass surgery	Milk-Alkali syndrome
Celiac disease	Multiple myeloma
Cleidocranial dysplasia	Osteogenesis imperfecta, type II
Clofibrate therapy	Pernicious or profound anemia
Cushing's syndrome	Radioactive heavy metals
Hypophosphatasia	Starvation
Hypothyroidism	Vitamin C deficiency
Improperly collected blood (oxalate, EDTA)[a]	Vitamin D intoxication
Inappropriate reference range	Wilson's disease
Massive transfusion	Zn^{++} or Mg^{++} deficiency

[a]*EDTA, ethylenediaminetetraacetic acid.*

Rarely, newborns with severe osteogenesis imperfecta (type II) can have low serum ALP activity,[107] as do some patients with *RUNX2* (*CBFA1*) deactivation causing cleidocranial dysplasia from quiescent OB function.[108] To assess these "hypophosphatasemias" vis-a-vis HPP, assay of plasma PLP (vitamin B_6) can help because an elevated level is a sensitive and specific marker for HPP.[2] I expect elevated PLP levels only in HPP because all TNSALP isoenzyme activity (including liver, not just bone) is reduced. Finally, a few reports of HPP describe transient increases in serum ALP activity (probably the bone isoform of TNSALP) after orthopedic surgery or fracture.[65] In theory at least,[95] conditions that increase circulating levels of any type of ALP (e.g., pregnancy, hepatobiliary disease) could mask the biochemical diagnosis of HPP.[34] Accordingly, if a puzzling patient is encountered, documentation that serum ALP activity was consistently low or at one time high or normal can be very helpful. Also quite useful is to determine if hypophosphatasemia or borderline-low serum ALP is present in first-degree relatives. Spontaneous mutation of *TNSALP* is rare, and therefore this information is frequently revealing. Quantitation of serum ALP isoenzymes, or specifically the bone TNSALP isoform, may hypothetically help in exceptional circumstances (e.g., pregnancy, certain malignancies, hepatobiliary disease), but normal ranges are not well established. Now, mutational analysis of the TNSALP (ALPL) gene is available from certified fee-for-service laboratories, and positive results can also support (but do not make) the diagnosis of HPP (see later).

4.3.1.2 Minerals

In contrast to nearly all types of rickets or osteomalacia, serum Ca or P_i levels are not low in HPP. In fact, the pathogenetic block of their entry into the skeleton in HPP caused by extracellular accumulation of PP_i (ePPi) can lead to a particularly striking and unique disturbance of Ca and P_i homeostasis when HPP is severe. In infantile HPP, hypercalcemia occurs with physiologically suppressed circulating PTH and 1,25-dihydroxyvitamin D levels.[80] Hypercalciuria is expected in this circumstance. In childhood HPP, only exceptional patients have mild hypercalcemia, but hypercalciuria is relatively common. Serum 25-hydroxyvitamin D levels are typically unremarkable.[109,110] Years past, such findings were attributed to an abnormality in the Ca-PTH feedback system,[114] and several HPP patients were reported with elevated serum PTH levels. However, renal compromise from hypercalcemia with retention of immunoreactive PTH fragments and rare occurrences of primary hyperparathyroidism[99] may have been the explanation.[2] Notably, however, patients with childhood or adult HPP are typically eucalcemic, yet they have serum P_i levels above the mean value for age-matched controls, and ~50% are distinctly hyperphosphatemic. Enhanced renal reclamation of P (increased tubular maximum for P/glomerular filtration rate; that is, TmP/GFR), but not from the block of Ca entering the skeleton suppressing PTH, underlies this finding.[93,115] Only sometimes is there accompanying suppressed circulating PTH. Our ongoing assessments of circulating FGF23 levels in HPP are showing results in the normal range (unpublished). Hence, TNSALP may actually function directly in renal excretion of P_i. Alternatively, excess urinary PP_i in HPP may mediate this disturbance. Notably, patients with generalized arterial calcification of infancy, GACI-1 (OMIM #208000)[15] featuring low extracellular levels of PP_i,[116] can develop hypophosphatemia and rickets, but reportedly with elevated circulating FGF23 levels.[117] Especially rare "HPP" patients have been described who are inexplicably hypophosphatemic from renal P_i wasting,[118,119] but *TNSALP* mutation and substrate studies were not yet available to establish this diagnosis.

4.3.1.3 Routine Studies

Other routine biochemical tests, including serum parameters of liver or muscle function (e.g., bilirubin, aspartate aminotransferase, lactate dehydrogenase, creatine kinase, aldolase), are unremarkable in HPP. Serum acid phosphatase activity is generally normal,[120] but OC-derived tartrate-resistant acid phosphatase was inexplicably elevated for more than a decade in one affected woman.[121] Increased levels of proline in blood and urine have been described in a few patients, but the significance is not known.[122] Bone turnover markers have not yet been detailed in published reports.

4.3.1.4 TNSALP Natural Substrates

An elevated level of PEA in blood or urine supports a diagnosis of HPP.[123] PEA is typically assayed in "inborn error" laboratories as a component of quantitative amino acid chromatography, but can be ordered specifically from a few commercial laboratories. However, phosphoethanolaminuria is not pathognomonic of HPP and can occur in other disorders, including several metabolic bone diseases.[124] Ideally, a 24-h urine collection is assayed and the PEA level "normalized" to the creatinine content. Importantly, PEA excretion in urine is conditioned by subject age and diet, follows a circadian rhythm, and can be normal in mild HPP.[64] The following age-adjusted reference ranges, expressed as micromoles of PEA per gram of urine creatinine, have been published (<15 years, 83–222; 15–30 years, 42–146; 31–41 years, 38–155; and >45 years, 48–93).[124]

My experience in HPP has been that an increased plasma level of PLP is a more sensitive and more specific marker compared to elevated serum or urine PEA.[7,102,125] There is, however, some recent contrary evidence in carriers or mildly affected adults.[113] PLP assays are readily

available in fee-for-service laboratories and typically ordered as "vitamin B_6." Generally, the more severe the HPP, the greater the PLP elevation.[2,7] Even patients with odonto-HPP manifest this finding.[7] Nevertheless, overlap of plasma PLP levels occurs from one clinical form of HPP to the next. To exclude false positive PLP elevations, supplements containing vitamin B_6 must not be taken for 1 week before testing.[125] Notably, assaying PLP in plasma obtained 1 day after pyridoxine hydrochloride is given orally daily for 6 days distinguishes HPP patients especially well because of their markedly increased level. In 1990, this proved helpful, first for identifying Mennonite carriers of severe HPP in Canada,[126] and then for our many HPP patients and carriers (unpublished).

Assay of inorganic pyrophosphate (PP_i) currently remains a research procedure. Plasma levels of PP_i are typically high-normal or elevated when skeletal disease is from HPP.[80,84] Urine levels of PP_i are increased in most HPP patients,[2] but occasionally values are unremarkable in mildly affected individuals.[39] Nevertheless, quantitation of urine PP_i has also been reported to be a sensitive way to detect HPP carriers.[127]

4.4 Radiological Findings

Radiographic survey of the skeleton reveals pathognomic changes in perinatal and infantile HPP (Figs. 28.1 and 28.2) and in severe childhood HPP (Figs. 28.5 and 28.6). The findings in adult HPP should suggest the disorder if recurrent metatarsal stress fractures preceded characteristic femoral pseudofractures (Fig. 28.7). However, the overall radiologic changes in adults are not diagnostic.

Bone scanning can reveal fractures, and may help to detect craniosynostosis.[83] Magnetic resonance imaging is necessary to identify the unusual painful bone marrow edema syndrome in HPP that can resemble chronic recurrent multifocal osteomyelitis or malignancy.[88]

Dual energy X-ray absorptiometry (DXA) in HPP may be difficult to fully interpret when there is short stature, deformity, or heterogeneous skeletal mineralization. To help, in 2012 we published simple equations for preteenage children to "correct" DXA bone mineral density values for stature by first calculating and then substituting patient's "height–age."[128]

4.5 Histopathological Findings

Primary histological abnormalities in HPP are observed in the hard tissues (skeleton and teeth). Secondary changes due to HPP complications occur elsewhere and in severe cases include extramedullary hematopoiesis occasionally noted in the liver,[43,85] and perhaps hypoplastic lungs.[79] Biopsy of weak muscle is essentially unremarkable.[2,49]

4.5.1 Skeleton

Except in odonto-HPP,[65] nondecalcified sections of bone following in vivo tetracycline labeling of the patient[43,85] show defective skeletal mineralization. This includes excesses of unmineralized skeletal matrix (osteoid) that can occur in a patchy distribution in adults[129] and in children.[130] Impaired skeletal mineralization is confirmed when fluorescence microscopy fails to show sufficient numbers of separate discrete fluorescent bands on bone surfaces where calcification should be proceeding at "mineralization fronts." Some questionable cases of pseudo-HPP lack this critical information from tetracycline administration. Unmineralized osteoid accumulates in HPP because it does not calcify properly for subsequent remodeling. Features of secondary hyperparathyroidism are typically absent in HPP, such as peritrabecular fibrosis, which occurs in other rickets or osteomalacia when there is hypocalcemia and secondary hyperparathyroidism. In HPP physes (growth plates), rachitic changes[43,85] can include disruption of the normal columnar arrangement of chondrocytes, widening of the zone of provisional calcification, and failure of primary spongiosa to calcify near degenerating cartilage cells. However, the sources of the bone isoform of TNSALP (chondrocytes and OBs, as well as their MVs) are present,[131] although with reduced TNSALP activity.[43] In some patients, OBs appear flat and inactive.[85] ALP levels in bone tissue correlate inversely with the degree of osteoid accumulation.[85] The numbers and morphology of OBs and OCs, as well as the appearance of unmineralized osteoid, vary from patient to patient. Woven bone, a finding that can reflect either bone repair following a fracture or defective skeletal formation, may be observed.[88] The severity of the mineralization defect in HPP generally reflects the clinical outcome.[85] In lethal HPP, even the bony structures of the middle ear can be poorly ossified. However, unless histochemical studies of ALP activity are performed, the light microscopy changes may be difficult to distinguish from other forms of rickets or osteomalacia.[88] Cranial "sutures" that appear widened on radiographs are not fibrous tissue, but are an illusion due to hypomineralization of the calvarial bones.[47]

Electron microscopy of perinatal and infantile HPP bone obtained at autopsy has revealed normal distribution of proteoglycan granules, collagen fibers, and MVs.[43,85] The MVs are deficient in ALP activity, yet contain HA crystals.[42] Accordingly, "primary" (phase 1) mineralization of the skeleton[43] seems intact in HPP. Importantly, however, only isolated or tiny groups of HA crystals (calcospherites), frequently not associated with MVs, have been observed.[43,77] This finding suggests extravesicular growth of HA crystals during "secondary" (phase 2) mineralization is compromised. Thus, these observations are consistent with pathogenic extracellular accumulation of PP_i in HPP (see later).[1,2]

4.5.2 Dentition

Premature loss of deciduous teeth occurs in several disorders including toxicities, metabolic errors, and malignancies (Table 28.2).[87] In HPP, this complication results from hypomineralization of partially acellular cementum covering tooth roots,[86] and varies in severity from tooth to tooth. Incisors are most vulnerable. Desiccated deciduous teeth may still be useful for examination. Any cementum present is afibrillar.[131] The number of teeth lost prematurely (see later) generally reflects the severity of HPP captured by the expanded nosology for pediatric patients.[74] Big pulp chambers in HPP suggest retarded dentinogenesis. Dentin tubules may be enlarged although reduced in number. The excessive width of predentin, increased amounts of interglobular dentin, and impaired calcification of cementum seem analogous to the osteoidosis observed in HPP bone. Conflicting reports discuss whether enamel is directly compromised.[87] The histopathological changes in the permanent teeth seem similar, but more mild.[89,131]

TABLE 28.2 Causes of Premature Loss of Deciduous (Primary) Teeth

I. Trauma:	
A.	Accidental
B.	Psychotic individuals
C.	Radiation exposure
II. Genetic:	
A.	Acatalasia
B.	Chediak–Higashi syndrome
C.	Chronic neutropenia
D.	Cyclical neutropenia
E.	Dentin dysplasia
F.	Hypophosphatasia
G.	X-linked hypophosphatemic (vitamin D resistant) rickets
H.	Lesch–Nyhan syndrome
I.	Papillon–Lefèvre syndrome
III. Neoplasms:	
A.	Lymphomas and leukemias
B.	Soft and hard tissue, benign and malignant, neoplasms either primary or metastatic
C.	Some tumor-like diseases.
IV. Miscellaneous:	
A.	Acrodynia
B.	Langerhans cell histiocytosis (histiocytosis X)
C.	Odontodysplasia (ghost teeth)
D.	Osteomyelitis
E.	Periodontitis
F.	Trisomy 21 (down syndrome)
G.	Vitamin C deficiency (scurvy)

4.6 Biochemical and Genetic Defect

4.6.1 TNSALP Deficiency

Early on, necropsy studies of affected newborns and infants with HPP identified the enzymatic defect and thereby predicted its genetic etiology. Profound deficiency of ALP activity was documented in skeletal, hepatic, and renal tissue yet ALP activity was normal in the intestine and placenta (fetal trophoblast).[132–134] This observation matched results emerging from amino acid sequence analyses of proteolytic peptide digests of ALPs purified from healthy human tissues[3] and indicated that HPP was an enzymopathy compromising all isoforms within the TNSALP isoenzyme family. Investigation of the hypophosphatasemia of HPP supported these studies by showing deficient activity in serum of both the bone and the liver isoforms of TNSALP[29] and no enhanced clearance of circulating TNSALP in HPP.[82] This was demonstrated during the 1980s with early attempts at enzyme replacement therapy for HPP (see later).[82] When administered intravenously to infants with life-threatening HPP, the bone isoform of TNSALP in plasma from patients with Paget's bone disease, and ALP extracted from healthy human placentas, had unremarkable circulating half-lives. Furthermore, coincubation studies of sera, as well as coculture of dermal fibroblasts excluded an inhibitor or absence of an activator of TNSALP in HPP.[47,65] Instead, the hypophosphatasemia of HPP reflected little bone or liver TNSALP activity entering the circulation. In 1984, fibroblast heterokaryon complementation studies implicated a defect at a single but unknown gene locus causing HPP (see later).[135]

Leukocyte ALP combines TNSALP and placental ALP and, therefore, can be low in HPP, except pseudo-HPP.[85] However, during HPP pregnancies, low leukocyte ALP activity may correct due to more placental isoenzyme.[136]

In 1989 and 1990, preliminary observations using a polyclonal antibody to the liver isoform of TNSALP indicated normal amounts of TNSALP in HPP tissues.[60] However, in 1996, monoclonal antibody-based immunoassays for serum dimeric TNSALP demonstrated low levels of bone and liver TNSALP in all forms of HPP except pseudo-HPP.[137] It seemed early on that upon release of TNSALP from cell surfaces, its circulating immunoreactivity was reduced in HPP.[114]

In autopsy material representing liver, bone, and kidney and in skin fibroblasts in culture from infants

with HPP, sensitive assays showed some ALP activity.[138] However, such fibroblast ALP usually had distinctive physicochemical properties.[138] In 1968, small bowel mucosa from a family with clinically mild HPP,[139] and tissues from severely affected patients, contained increased intestinal ALP. In one patient, inhibition of catalysis and isoelectric focusing in 1983 suggested intestinal ALP[133] was present perhaps by compensatory expression of this ALP isoenzyme. However, in 1996, perinatal and infantile HPP fibroblasts in culture showed ALP with physicochemical properties like TNSALP,[140] but these and immunological characteristics differed from patient to patient.[140]

Autopsy studies of children or adults with HPP have not been reported,[141] but globally diminished TNSALP activity became apparent from studies of the activity of its isoforms in serum,[29] circulating granulocytes, bone biopsy specimens,[85] and cultivated skin fibroblasts.[140]

4.6.2 Genetic Defect in HPP

Our investigation in 1984 of HPP skin fibroblast heterokaryons representing 10 unrelated families with perinatal or infantile HPP showed no "complementation" (i.e., no correction of low-ALP activity in the cell homogenates) implicating a single gene locus causing HPP.[135]

In 1987, genetic linkage of the RH blood group to perinatal HPP in six Mennonite kindreds in Manitoba, Canada bolstered the "candidacy" of the TNSALP gene.[142]

In 1988, characterization of the gene encoding TNSALP[20,143] was followed that year by discovery of a homozygous, loss-of-function, missense mutation in *TNSALP* in a Canadian boy with perinatal HPP.[3] The three-dimensional structure of *Escherichia coli* ALP[25] suggested his mutation compromised the spatial relationship of metal ligands to an important arginine residue at the catalytic pocket. Later, his mutation was also shown to impair the biosynthetic transport and cause intracellular aggregation of his TNSALP. Then, in 1992, four unrelated American perinatal or infantile HPP patients were found to carry compound heterozygous TNSALP missense mutations[143] that perhaps disrupted metal ligand binding.[23] Also, two siblings with childhood HPP and one unrelated woman with adult HPP proved compound heterozygotes for identical *TNSALP* missense mutations, showing that relatively mild HPP can be an AR disorder.[143] In 1993, homozygosity for a unique "founder" *TNSALP* missense mutation accounted for perinatal HPP in Canadian Mennonites.[57] A different founder defect is present among the Japanese.[62] Now, more than 330 different *TNSALP* mutations have been reported in HPP patients worldwide,[4] including missense, nonsense, and donor splice site mutations, and frame shift deletions.[3,57,59,61,62,143–145] Notably, ~80% of these mutations are missense.[4] Except in Mennonites in Manitoba, Canada and in the Japanese, homozygosity rarely explains HPP. Accordingly, molecular investigation of new HPP cases typically requires mutational analysis of the splice sites and coding exons of *TNSALP*, but deletion analysis is less likely to be abnormal.

Transfection studies have now shown that certain *TNSALP* mutations: (1) diminish expression of the mutated allele, (2) compromise mRNA stability, (3) inactivate the catalytic site, or (4) sequester the enzyme intracellularly.[59,145] Some *TNSALP* mutations exert dominant-negative effects,[55,145] and thereby explain AD transmission of HPP (see later). Two such defects account for relatively many instances of AD HPP in the United States.[146]

Diagnosing HPP is usually straight forward, especially when severe, without *TNSALP* mutation analysis. This genetic information is, however, critical for documenting inheritance patterns, establishing recurrence risks, and for prenatal assessment when requested.[71] From my experience, I expect all patients with HPP to carry one or two defective *TNSALP* alleles. Thus far, there is no genetic heterogeneity for HPP explained by another causal gene. Only rarely are these mutations difficult to identify. All clinical forms of HPP, including pseudo-HPP,[73] originate from loss-of-function mutation(s) in *TNSALP*.

4.6.3 Inheritance

The first evidence that HPP is heritable[47] came soon after Rathbun's 1948 publication.[44] Affected siblings were reported in 1950.[147] Early on, family studies of severe HPP in infants or children indicated AR inheritance. The parents often had low or low-normal levels of serum ALP activity, and PEA could be detected in their urine.[46,47]

TNSALP mutation analysis has shown that perinatal and infantile HPP reflect AR inheritance.[55,80] Odonto-HPP is predominantly AD.[55] Adult HPP and mild or severe childhood HPP can be AD or AR.[55] Nevertheless, even odonto-HPP can rarely be inherited as an AR disorder.[55] In fact, early reports described childhood and adult HPP, as well as odonto-HPP, as AR conditions because vertical transmission of clinically apparent HPP seemed unusual.[65,66] Then, multigenerational occurrences of clinical HPP were increasingly recognized, and indicated that these relatively mild forms of HPP can be AD as well as AR diseases.[65,66,89,148,149] Also, family studies sometimes documented mild HPP in individuals who had severe AR HPP in their offspring.[65,150,151]

Early on, identification of carriers of HPP necessitated quantitation of several of its biochemical markers including urine PP_i.[152] Pyridoxine loading, followed by assay of plasma PLP levels, was helpful for heterozygote detection in the Mennonite population in Canada.[126]

4.6.4 Epigenetic and Nongenetic Effects

Sometimes, considerable differences in HPP severity manifest among siblings despite identical *TNSALP*

genotypes. There is, however, no evidence that *TNSALP* is imprinted. Accordingly, other genes and nongenetic factors can apparently condition HPP expressivity. In fact, in 1986 one remarkable patient with infantile HPP suggested dysregulation of TNSALP biosynthesis. Following attempted treatment using prednisone and PTH (1–34) and then a series of intravenous infusions of pooled plasma from healthy individuals,[153] he showed transient 4-month correction of his hypophosphatasemia accompanying remineralization of his skeleton and clinical and radiographic improvements with enhanced skeletal synthesis of the bone isoform of TNSALP.[153] However, his remarkable but transient improvement despite subsequently identified underlying homozygous *TNSALP* missense mutations remains a mystery.[154]

Physiological regulation of TNSALP biosynthesis may also impact HPP. Patients with childhood HPP usually have higher absolute levels of serum ALP activity compared to adult-onset cases. Their hypophosphatasemia seems to undergo further important decreases during skeletal maturation. Possibly, their physiological decrease in skeletal ALP, reflected in serum ALP activity,[13] explains why some adults manifest HPP who were previously considered "carriers." Perhaps, senescence of OBs with aging also contributes to adult-onset HPP.

Investigation of benign prenatal HPP has suggested mechanical factors (fetal packing) and the biochemical environment of a mother carrying a defective *TNSALP* allele can adversely affect her fetus with HPP.[71]

It is unknown if dietary mineral levels condition HPP severity. Possibly, excessive dietary Ca suppresses PTH production thereby diminishing TNSALP biosynthesis by OBs. Excessive intake of P_i could increase extracellular levels of this competitive inhibitor of TNSALP.[1,2]

4.7 Treatment

4.7.1 Prognosis

Prior to experimental bone-targeted TNSALP-replacement therapy (asfotase alfa) for HPP[80] (see later), perinatal HPP was almost always rapidly fatal.[75] Only rarely was there prolonged survival. Hence, it is crucial to distinguish benign prenatal HPP, that shows spontaneous improvement after birth, from perinatal HPP (see previously).[71] The outcome of infantile HPP cannot be predicted when the patient is first evaluated.[1,2] Sometimes there is progressive skeletal deterioration that leads to death from pneumonia within a few months.[82] In others, significant spontaneous improvement may occur,[155] or they may suffer persisting rachitic disease[54] including craniosynostosis.[51] Once infantile HPP is diagnosed, close sequential clinical assessments with radiographic studies are crucial for prognostication. Although the precise likelihood is not known, perhaps 50% of patients with infantile HPP die from respiratory compromise and pneumonia that follows worsening skeletal disease of the chest.[1,2,47] In others, there may be significant improvement, particularly after infancy, perhaps because growth rates decrease and any residual TNSALP activity becomes more effective in mineralizing the skeleton. Indeed, a preliminary report in 1986 from Canada suggested that the adult stature of survivors of infantile HPP can be normal,[155] but there are significant exceptions both there and in the United States. Childhood HPP may also seem to improve spontaneously when growth plates fuse in young adult life,[47] but recurrence of symptoms and complications later is possible, if not likely.[113]

4.7.2 Supportive

Severely affected infants and young children with HPP should be followed carefully to detect neurological complications, such as increased intracranial pressure, from either "functional" or "true" craniosynostosis.[51,80,84] Functional craniosynostosis can occur despite the radiographic illusion of widely open fontanels, and may require craniotomy.[76,80] In other circumstances, skull deformity may occur but without neurological problems.

Vitamin B_6-dependent seizures manifest only in severe HPP (perinatal or infantile forms), but represent a grave prognostic sign,[50] probably because TNSALP deficiency must be especially profound to block hydrolysis of PLP to pyridoxal for neurotransmitter synthesis (see later).

Fractures in children with HPP do mend, although delayed healing seems likely and has occurred after femoral osteotomy with casting. In adult HPP, proximal femoral pseudofractures may remain unchanged for years, but will not unite unless they complete or receive prophylactic intramedullary fixation.[90,91,100] Load-sharing intramedullary rods or nails, rather than load-sparing plates, and so on, seem best for management of pseudofractures or acute femoral fractures.[91] For recurrent metatarsal stress fractures, ankle-foot orthoses may help.

Expert dental care is important for HPP. Tooth loss in children can impair speech and nutrition. Rarely, preservation of their teeth or complete or partial dentures become necessary.[86] Bacteria on the tooth surface, perhaps related to TNSALP deficiency in leukocytes, possibly contributes to tooth loss.

Symptoms from CPPD or Ca–P_i crystal deposition may respond to nonsteroidal antiinflammatory medication.[96] One report,[156] and our own experience, indicates that naproxen is useful for pain in children with HPP, including during the unusual syndrome of bone marrow edema.[88]

Prior to 2015 (see later), there was no approved medical therapy for HPP, although a variety of treatments had been attempted.[47,65,151,157,158] Traditional regimens for rickets and osteomalacia (vitamin D and mineral

supplements) are avoided unless deficiencies are documented, because circulating levels of Ca, P_i, and the vitamin D metabolites are not low.[80,84] In infantile HPP, excessive vitamin D or mineral supplementation could provoke or exacerbate any hypercalciuria or hypercalcemia. Conversely, restriction of dietary vitamin D or sunshine exposure should be avoided because superimposed vitamin D-deficiency rickets has occurred in HPP.[110]

Hypercalcemia in infantile HPP can improve with lowering dietary Ca intake and/or with hydration, loop diuretics, or glucocorticoid therapy.[76,80,82] Progressive skeletal demineralization can follow, but is probably due to the HPP per se if serum levels of Ca and P_i do not become low.[82] Synthetic salmon calcitonin[158] or aminobisphosphonates[159] to block skeletal mineral loss are not useful for hypercalcemia in HPP. In fact, concern for aminobisphosphonate exposure in HPP came in 2012 with report of a woman who was a HPP carrier or early in the course of adult HPP who sustained "atypical subtrochanteric femoral fractures" after receiving alendronate and then zoledronate for "osteoporosis."[100] Bisphosphonates could cause harm in HPP because they are analogs of PP_i, perhaps further impairing skeletal mineralization, lower bone turnover more, or inhibit ALPs by binding Zn^{++} and Mg^{++}.[100,116] Adult HPP is a chronic bone disease after the onset of symptomatology.[65,66,91,113] Affected woman may suffer worsening osteomalacia at menopause leading to pain and fractures, but this does not seem preventable by estrogen replacement therapy (personal observation).

4.7.3 *Experimental*

In the 1950s, administration of cortisone to a few patients with severe HPP reportedly was followed by periods of normalization of serum ALP activity and radiographic improvement,[47,160] but this has not been a consistent finding.[47] Subsequently, brief treatments with Zn^{++} or Mg^{++} were unsuccessful.[65]

Because increased ePPi is a major factor for the skeletal disease of HPP (see later),[7] reducing the level should help skeletal mineralization to proceed.[34,39] In 1968, oral P_i supplementation hoping to promote renal PP_i excretion reportedly had some radiographic success.[161] However, subsequent studies showed plasma PP_i levels were unchanged. In fact, increased urinary PP_i levels after P_i is administered may instead reflect enhanced renal synthesis rather than excretion of PP_i.[34,39] Thus, P_i supplementation has not been pursued.[9,133] Indeed, hyperphosphatemia is common in HPP,[1,2,120] and P_i is a competitive inhibitor of ALP.[13]

Early on, enzyme replacement therapy for infantile HPP was attempted by intravenous infusions of different soluble human ALPs, but was generally disappointing.[82,162] In 1982, this involved plasma rich in the bone isoform of TNSALP obtained from two patients with Paget's bone disease and was associated with slight radiographic improvement, but still a lethal outcome.[82] That same year, fresh plasma given weekly for one patient was accompanied by clinical and some radiographic improvement. However, in 1984, three infants showed no significant clinical or radiographic benefit using Paget plasma infusions and succumbed to severe HPP.[157] In 1986, intravenous infusions of plasma pooled from several healthy individuals preceded transient correction of hypophosphatasemia with marked temporary clinical, radiographic, and histological improvement in one severely affected boy with HPP (see aforementioned).[153,154] However, in 1988, a similar trial in a different patient did not reproduce this response. In 1989, a brief report suggested that liver TNSALP improved the histological appearance of bone and decreased urinary PEA levels in one patient.[163] In 1992 in a preliminary report,[162] ALP purified from a human placenta caused hyperphosphatasemia but led to only modest decrements of plasma PLP and urinary PEA concentrations, no change in urinary PP_i levels, and no clinical or radiographic improvement for lethal HPP. This approach was based upon our observation that would be reported in 1995 that placental ALP was catalytically active in vivo toward PEA, PP_i, and PLP in a study of pregnant carriers for HPP.[164] These cumulative observations suggested that the amount of ALP required for successful enzyme replacement therapy would be much greater than the levels achieved in the circulation by these treatment attempts, or perhaps ALP needed to be on cell surfaces, particularly in the skeleton, to act therapeutically.[162] In this regard, it is notable that the extreme skeletal disease of perinatal HPP occurs in utero but is not prevented by the mother's substantial circulating placental ALP during the third trimester. Instead, stimulation of TNSALP biosynthesis or enhancement of the enzyme's activity, especially in the skeleton, might be beneficial. Of interest, Mabry syndrome (OMIM #239300),[15] which features *hyperphosphatasemia* and mental retardation, involves impaired binding of ALPs and many other proteins to plasma membranes yet is not associated with rickets/osteomalacia.[20]

Then, in 1996, preliminary findings in the TNSALP gene knock-out mouse supported marrow cell transplantation as a means to increase directly ALP activity near skeletal matrix in HPP.[165] Hence, for two unrelated girls likely to die from infantile HPP, marrow cell and bone cell transplantation in 2003 and 2007, respectively, seemed beneficial although donor cell engraftment was low.[166] In 2009, allogeneic mesenchymal stem cell transplantation to become OBs had some benefit for a patient with perinatal HPP.[167] In 2011, TNSALP delivered by lentiviral gene therapy had success in TNSALP knockout mice.[168]

Beginning in 2006, injections "off label" of the active fragment of PTH (1–34) or the intact PTH molecule (1–84) to stimulate bone TNSALP biosynthesis reportedly helped some patients with adult HPP[93,169] including reduction of bone pain and healing of pseudofractures or bony nonunions.[93,170] Perhaps PTH administration is most useful for AD HPP when one healthy *TNSALP* allele is present and can be overexpressed.[93]

4.7.4 Prenatal Diagnosis

Assay of serum ALP in utero using cord blood (cordocentesis) is untested for diagnosing HPP. Quantitation of α-fetoprotein in amniotic fluid helps differentiate anencephaly from severe HPP. Historically, measuring ALP activity in amniotic fluid is not useful for diagnosing HPP[171,172] because at 14–18 weeks gestation the level derives primarily from intestinal ALP excreted from the fetus.[172] Furthermore, first trimester chorionic villus samples were studied utilizing a monoclonal antibody-based assay specific for TNSALP, but this approach was halted.[173] In 1990, restriction fragment length polymorphism analysis using a chorionic villus sample was successful for assessing HPP in a Canadian Mennonite family,[174] and in 1991 helpful for a Japanese family.[175]

Several early reports considered for fetuses with HPP that identification of skeletal changes by radiologic techniques indicated a lethal outcome. During the second trimester, perinatal HPP has been diagnosed from ultrasonography (with attention to the limbs, as well as to the skull), radiography, and assay of ALP activity in amniotic fluid cells.[171] However, ultrasonography was judged to be normal at 16–19 weeks of gestation in three cases of perinatal HPP in which radiographic studies near term showed absence of a fetal skeleton.[176] Combined use of radiological techniques, including serial ultrasonography, seemed best. Importantly, however, our experience published in 2011[71] with benign perinatal HPP has led us to conclude that routine ultrasonography cannot diagnose lethal HPP in utero early in pregnancy.

Since 1995, *TNSALP* mutation analysis has evaluated pregnancies at risk for HPP.[177] Molecular assessment of *TNSALP* is now available in several commercial laboratories. Although usually not needed for postnatal diagnosis of HPP, the information is critical for understanding the inheritance pattern of HPP and for prenatal assessments (typically when there has been a previously affected sibling with severe disease). However, characterization of the benign prenatal form of HPP has raised important issues concerning the predictability of the outcome especially for fetuses with defects in both *TNSALP* alleles.[71] In fetuses with benign perinatal HPP, bowing has corrected spontaneously late in the pregnancy, as well as postnatally, with the clinical phenotype otherwise then ranging from infantile HPP to odonto-HPP.[71]

4.8 Mouse Model for Hypophosphatasia

In 1995 and 1997, separate laboratories reported homologous recombination that inactivated the equivalent of the TNSALP gene in mice, and then created a murine knock-out model that recapitulated infantile HPP extremely well.[178] Their bones were unremarkable at birth.[178] Subsequently, they developed pyridoxine-dependent seizures apparently from diminished hydrolysis of PLP to pyridoxal for gamma-aminobutyric acid synthesis in the brain.[50] Parenteral administration of vitamin B_6 only briefly prolonged their lives.[178] Concomitantly, there was defective skeletal mineralization, as well as endogenous accumulation of PEA, PP_i, and PLP.[178] In 2008, the murine model became key in preclinical studies for demonstrating the efficacy of bone-targeted enzyme-replacement therapy (asfotase alfa) for HPP,[179] including prevention of the skeletal, dental,[180] and neurological manifestations.

In 2007, an *N*-ethyl-*N*-nitrosourea-based murine model of mild HPP was reported that exhibited late-onset skeletal disease featuring defective endochondral bone mineralization that led to arthropathies of the knees and shoulders.[181] Then, further murine models featuring relatively mild HPP were generated.[182,183]

5 ENZYME REPLACEMENT THERAPY FOR HPP

HPP was the last rickets/osteomalacia to await a medical treatment.[54] Then, in 2008, patient trials began that evaluated an experimental, recombinant, HA-targeted,[180] TNSALP replacement therapy designated asfotase alfa (AA).[5] AA is a fusion protein that consists of multimeric TNSALP, the Fc fragment of immunoglobin G_1, and a deca-aspartate motif for mineral targeting.[5,80,179] In 2012, the 1-year experience was published concerning nine infants or young children who had received AA treatment because of life-threatening perinatal or infantile HPP.[80] Their bone mineralization assessed radiographically (Fig. 28.8) improved, sometimes within several weeks, and was followed by better pulmonary, cognitive, and motor function.[80] The continuing favorable experience, now representing 7 years of treatment, has been reported in preliminary communications.[184,185] Soon after, AA treatment began evaluation for children with severe childhood HPP.[84] They suffered weakness, functional impairments, and pain, and had substantial rickets that could be assessed longitudinally using radiographs of their wrists and knees.[84] Their bone health with AA treatment was also assessed by stature and physical function. Muscle strength improved with resolution of pain and disability and without resistance to this biologic that showed a good safety profile.[84] In 2016,

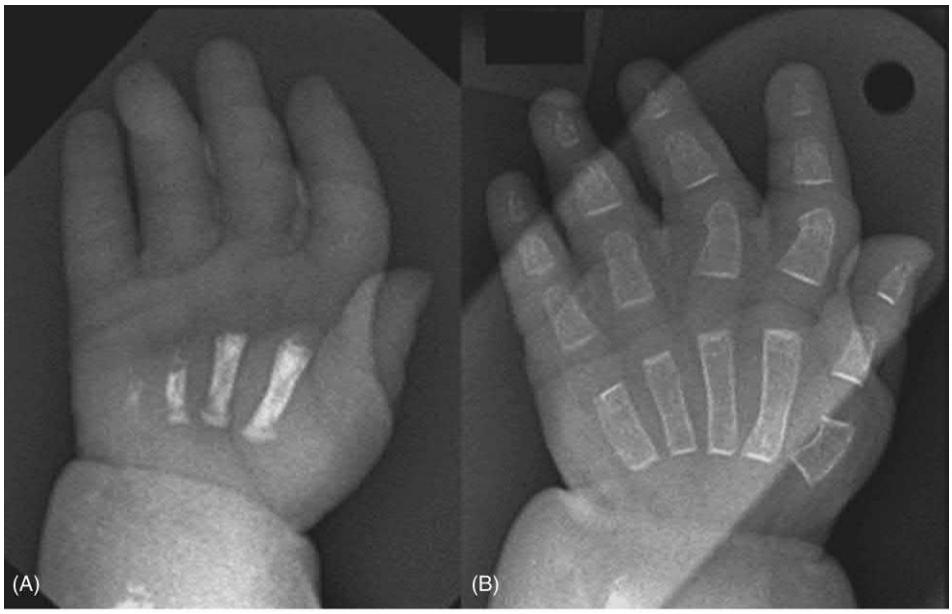

FIGURE 28.8 (A) At baseline, before bone-targeted TNSALP-replacement therapy (asfotase alfa) for perinatal hypophosphatasia, there is extreme hypomineralization of the left hand. (B) Remarkable mineralization is apparent at Week 24 of therapy. *Source: Reproduced with permission from the N E J Med 366: 904–913, 2012.*

the findings for the children were detailed at 5 years of treatment.[84] With AA therapy, circulating levels of PLP and PP_i diminished, and anti-AA antibody levels were low with no evidence they caused resistance to this treatment.[80,84] Now, clinical trials involving AA have included HPP patients of wide-ranging ages, including adults (ClinicalTrials.gov). In 2015, AA (Strensiq) was approved in Japan for HPP, and in Canada, the European Union, and the United States and elsewhere for pediatric-onset HPP.[54] The new challenges for clinicians from this advance were reviewed in 2017.[54] Among them are: (1) correctly diagnosing HPP recognizing that there is a differential diagnosis for hypophosphat*asemia*, including several bone disorders,[1,2,113] (2) appreciating HPP's extraordinarily broad-ranging severity and determining which patients are appropriate for AA treatment,[80,84] and (3) understanding and arranging for the therapy and its follow-up for safe and effective treatment.[54] In the future, mouse models for HPP now suggest that gene therapy using marrow cell transplantation or viral vectors carrying ALP may someday cure HPP.[186]

6 PHYSIOLOGICAL ROLE OF ALKALINE PHOSPHATASE EXPLORED IN HYPOPHOSPHATASIA

The purpose of the gene duplications in humans that led to the three tissue-specific ALPs remains unclear. Intestinal ALP may have a function in protecting the gut and in promoting lipid absorption.[187] Documentation beginning in 1988[3] that loss-of-function mutation(s) of the candidate gene for HPP, *TNSALP*, causes this inborn-error-of-metabolism[4] established after 65 years[6] that Robison's hypothesis was correct[33]; ALP is essential for skeletal mineralization. Although liberation of P_i from an unidentified phosphocompound was a key component of Robison's theory,[6] he also later recognized that a second and unknown factor controlled skeletal mineralization.[33] This proved to be ePPi, a potent inhibitor of HA crystal initiation and growth.[38] Then, in 1997, electron microscopy supported a fundamental pathophysiological disturbance in HPP[131]—a block in phase 2 skeletal mineralization of cartilage and bone occurring after HA crystals rupture their MVs. HA crystals were found appropriately in HPP MVs, but were absent nearby in the skeletal matrix.[131] The high levels of ePPi in HPP apparently stopped extravesicular HA crystal growth and thereby halted hard tissue mineralization. The dental defects in HPP seem analogous to those in the skeleton.[180] Although it has been suggested that TNSALP deficiency might diminish the biosynthesis of the phospholipid surfactant causing atelectasis in HPP, the pulmonary problems of severe HPP seem readily attributable to the associated muscle weakness, thoracic deformity, and rib fractures.[75,81] The liver and adrenals are rich in TNSALP,[4] but they do not seem to malfunction in HPP. Two Japanese siblings with infantile HPP died with sudden unexplained liver failure as teenagers, but hepatic function appears normal in HPP patients.[80,84]

Several studies have suggested that TNSALP functions in cell growth and differentiation, but

TNSALP-deficient infantile HPP fibroblasts proliferate normally in culture.[1,2] Also, two-dimensional gel electrophoresis revealed unremarkable profiles of plasma membrane-associated phosphoproteins, hence TNSALP does not seem to be a phosphoprotein phosphatase.[188] In fact, TNSALP in humans may have little physiological importance other than mineralization of hard tissues and controlling the bioavailability of circulating vitamin B_6.[1,2] However, multiple other roles have been suggested[5] including in calcification;[5,34] for example, ALPs have domains that predict binding of proteins (including types I, II, and X collagen) that could orient TNSALP in skeletal matrix for mineral deposition.[5]

As reviewed below, the discovery that PEA, PLP, and PP_i accumulate endogenously in HPP inferred that they are natural substrates for TNSALP. This has been substantiated using in vitro studies.[8] Identification of extracellular accumulation of PP_i and PLP in HPP has been critical for understanding the physiological role of TNSALP, and the biochemical pathogenesis of this inborn-error-of-metabolism.[1,2]

6.1 Phosphoethanolamine

The discovery in 1955 that urinary PEA is increased in HPP provided a useful second biochemical marker for this disorder and the first evidence of a natural substrate for TNSALP.[52,53,123] Kidney handling of PEA in healthy subjects showed excretion when plasma levels are scarcely detectable; that is, essentially no renal threshold for PEA.[123]

Although its metabolic origin is not certain, PEA is thought not to be derived from phosphatidylethanolamine; that is, not from plasma membrane phospholipid breakdown. Indeed, PEA is a component of the phosphatidylinositol-glycan linkage apparatus for many cell-surface proteins.[5,34] Accordingly, extracellular PEA could originate from these biological anchors. Alternatively, the major source of circulating PEA could be the liver,[169] which metabolizes PEA to ammonia, acetaldehyde, and P_i in a reaction catalyzed by O-phosphorylethanolamine phospholyase. It is noteworthy that this enzyme requires PLP as a cofactor. In fact, in one family with adult HPP,[20] urinary levels of PEA correlated inversely with the serum activity of the liver (but not the bone) isoform of TNSALP.[29] Years ago, it was proposed that pseudo-HPP might result from deficiency of this enzyme.[169]

6.2 Pyridoxal 5′-Phosphate

In 1985, discovery that plasma PLP is substantially elevated in HPP but that most patients show no evidence of vitamin B_6 toxicity or deficiency importantly clarified the physiological role of TNSALP.[7] In health, the dietary forms of vitamin B_6 (pyridoxine, pyridoxal, pyridoxamine, and their phosphorylated derivatives) are absorbed from the gastrointestinal tract and then are converted to PLP in the liver.[125] PLP is subsequently released into the circulation primarily coupled to albumin.[125] A fraction of plasma PLP is bound to various enzymes.[125] Only a small amount of PLP normally circulates freely. Like other phosphorylated compounds, PLP cannot traverse plasma membranes and must first be dephosphorylated to pyridoxal (PL).[125] After PL crosses the plasma membrane, it is rephosphorylated to PLP or converted to pyridoxamine 5′-phosphate to act intracellularly as a cofactor for many enzymatic reactions.[7] PLP is the major cofactor form of vitamin B_6.[1] Ultimately, "vitamin B_6" is degraded to pyridoxic acid, primarily in the liver, and excreted into the urine.[125]

Elevated PLP in HPP plasma and an understanding of vitamin B_6 metabolism indicated that TNSALP acts importantly in the extracellular dephosphorylation of PLP and likely other phosphocompounds.[7] In fact, when serum levels of the bone or the liver TNSALP isoforms are increased by other skeletal or hepatobiliary diseases, plasma PLP levels are decreased. Clinical scrutiny revealed that HPP patients typically do not have symptoms of vitamin B_6 deficiency, such as dermatitis, stomatitis, peripheral neuritis, depression, or anemia or of toxicity, such as peripheral neuropathy.[7] Furthermore, children with HPP respond normally when tested for vitamin B_6 deficiency by L-tryptophan loading (Whyte MP and Coburn SP: unpublished observation). Indeed, urinary concentrations of the vitamin B_6 degredation product 4-pyridoxic acid are normal in HPP[7] as are intracellular levels of PLP and total vitamin B_6 in homogenates of TNSALP-deficient patient fibroblasts in culture. Also, tissues obtained at autopsy from three patients with perinatal HPP, in whom plasma PLP concentrations were markedly elevated, had unremarkable levels of PLP, PL, and total vitamin B_6.[141] Therefore, from these observations it was deduced correctly that TNSALP functions as a cell-surface enzyme.[7,125]

Nephrocalcinosis or nephrolithiasis in severely affected infants with HPP,[80] despite any protection again Ca–P_i deposition from the excessive PP_i in their urine, is likely due to the overwhelming effects of their hypercalciuria. However, altered oxalate metabolism (a consequence of vitamin B_6 deficiency) has not been explored in HPP.

Only those HPP patients with the perinatal or infantile from of the disease have pyridoxine-dependent seizures due to low plasma PL. Of interest, however, PEA seemed epileptogenic when given intravenously in 1983 to an infant with HPP during a study of PEA metabolism.[189] The epilepsy of severe HPP in babies is now understood to be vitamin B_6 dependent.[50] Fortunately, in all but the most severely affected HPP patients, sufficient extracellular dephosphorylation of PLP to PL by some mechanism seems to explain their normal (or somewhat

elevated) plasma PL levels and account for their physiological vitamin B_6 status.[7,125] Subsequent characterization of TNSALP as a plasma membrane-bound, cell-surface glycoprotein, covalently linked to the polar head group of phosphatidylinositol,[190] supported this mechanism. Studies using dermal fibroblasts from patients with infantile HPP and human osteosarcoma cells in culture showed that TNSALP is indeed attached to plasma membranes with ectotopography, and dephosphorylates PLP and PEA at physiological concentrations and at physiological pH.[8]

6.3 Inorganic Pyrophosphate

The discovery in 1965 that PP_i levels in urine are increased in HPP patients[9] suggested a mechanism for the impaired HA crystal deposition and thus the defective skeletal mineralization.[10,39] At high concentrations, PP_i adsorbs to amorphous Ca–P_i and prevents its transformation to HA crystals.[10] Furthermore, adsorption of PP_i to HA crystals impairs their growth and dissolution.[38,39] Hence, PP_i accumulation extracellularly in HPP surrounding MVs would lead to rickets or osteomalacia.

Studies using TNSALP-deficient fibroblasts from perinatal and infantile HPP patients demonstrated that these cells generate PP_i at normal rates from extracellular ATP.[191] They have unremarkable activity of nucleoside triphosphate pyrophosphatase (NTP-PP_i-ase), also called ENPP1 or PC-1.[191] Furthermore, clearance studies of ^{32} PPi administered to adults with HPP indicated that the endogenous accumulation of PP_i results from defective degradation of PP_i rather than increased PP_i biosynthesis.[39]

Of interest, at certain concentrations PP_i can also enhance precipitation of Ca and P_i to form amorphous Ca–P_i.[39] Accordingly, the calcific periarthritis and ligamentous calcification of HPP[68] could perhaps reflect this action of PP_i.[96]

ALP has been shown to dissolve CPPD crystals in vitro.[26] Thus, CPPD deposition leading to chondrocalcinosis, pseudogout, and pyrophosphate arthropathy[66] likely results from failure of TNSALP to hydrolyze PP_i with its subsequent endogenous accumulation, as well as persistence of CPPD crystals once formed.

6.4 Alkaline Phosphatase in Serum

A variety of evidence indicates that circulating, soluble, dimeric ALP is physiologically inactive.[1,2] Infants with HPP given plasma rich in soluble ALPs intravenously (correcting or even elevating the level of ALP activity) demonstrated essentially no clinical or radiographic improvement.[157] Furthermore, this treatment attempt failed to substantially reduce urinary PEA or PP_i levels or plasma PLP concentrations.[157] Accordingly, deficiency of TNSALP activity within the skeleton itself seemed to account for the rickets or osteomalacia of HPP.[157] In fact, in 1955 Fraser et al.[52] reported that rachitic rat cartilage would calcify in the serum of an infant with HPP, but slices of the patient's costochondral junction would not mineralize in synthetic calcifying medium or in pooled serum from healthy children. Hence, TNSALP in bone and cartilage tissue is the physiologically active form of this enzyme.

6.5 Overview for Tissue-Nonspecific Alkaline Phosphatase Function

Studies of vitamin B_6 metabolism in HPP together with delineation of the disorder's clinical features revealed that TNSALP is a cell-surface enzyme. This was later confirmed by a variety of tissue and cell culture studies showing that ALPs bind to plasma membranes by glycophosphatidylinositol-anchoring. Increased levels of PEA, PP_i, and PLP endogenously in HPP indicate that TNSALP is active toward phosphocompounds with variable chemical structure. In fact, it seems quite possible that additional natural substrates for TNSALP await discovery. The source of extracellular PEA is unclear, but could be the glycophosphatidylinositol anchor itself. Accumulation of membrane-impermeable PLP in plasma, but unremarkable PL levels in the circulation and tissues, explains the absence of vitamin B_6 toxicity, whereas only profound deficiency of TNSALP compromises dephosphorylation of PLP to PL to cause vitamin B_6-dependent seizures. Generation of extracellular PP_i, perhaps from ATP by the action of NTP-PP_i-ase, or pumped from intracellular sources,[5] occurs normally in HPP. PP_i accumulation in HPP reflects decreased PP_i degradation.[176] In HPP, Ca–P_i crystal deposition as HA causes calcific periarthritis, and CPPD precipitation results in chondrocalcinosis and sometimes in PP_i arthropathy. Calcific periarthritis may reflect the effect of PP_i at certain concentrations to stimulate Ca–P_i precipitation. Chondrocalcinosis and PP_i arthropathy occur from the PP_i accumulation, as well as the failure of TNSALP to hydrolyze CPPD crystals. Rickets or osteomalacia develops in HPP due to the accumulation of ePPi at sites of skeletal mineralization. High concentrations of ePPi surrounding MVs inhibit HA crystal growth. ALP activity in proximity to fibrillar collagen dictates which tissues might mineralize,[192] and presumably explains why ALP-rich liver, intestine, and placenta do not calcify. TNSALP appears to be physiologically important locally where it is bound to tissues, but not when soluble in the circulation.

Since at least three phosphocompounds (PEA, PP_i, PLP) accumulate in extracellular fluid at nanomolar or micromolar concentrations in HPP, TNSALP acts at substrate concentrations that are much lower than those used in routine clinical or research assays to measure

ALP activity. It is clear, however, that TNSALP functions at physiological pH. Accordingly, the term "ALP" (never used by Robison) is memorable but misleading.

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ABBREVIATIONS

AD Autosomal dominant
ALP Alkaline phosphatase
ALPL Gene mapping symbol for the TNSALP locus
AR Autosomal recessive
CPPD Calcium pyrophosphate dihydrate
ePPi Extracellular inorganic pyrophosphate
HA Hydroxyapatite
HPP Hypophosphatasia
MV Matrix vesicle
OB Osteoblast
OC Osteoclast
OMIM Online mendelian inheritance in man
P Phosphorus
PEA Phosphoethanolamine
P_i Inorganic phosphate
PLP Pyridoxal 5′-phosphate
PP_i Inorganic pyrophosphate
PTH Parathyroid hormone
TmP/GFR Tubular maximum for P/glomerular filtration rate
TNSALP Tissue-nonspecific ALP isoenzyme
***TNSALP* (*ALPL*)** Gene that encodes TNSALP

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CHAPTER

29

Sclerosing Bone Disorders

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1 INTRODUCTION

Many recent advances have been made in our understanding of the mechanisms underlying bone remodeling and homeostasis. The elucidation of the genetic mechanisms of many rare bone dysplasias made a major contribution to the current knowledge concerning how osteoclasts and osteoblasts work and cross talk. The relationship between the molecular defects causing these aberrant bone phenotypes and the clinical findings, gave new insights about the molecular machinery of both cell types and how they affect bone density. Especially, the study of skeletal pathologies caused by a disturbance of the balance between bone formation and bone resorption has significantly advanced what we know. Most of these disorders with increased bone density are monogenic, thus proving that one gene can dramatically influence bone balance either by impairing bone resorption or by increasing bone formation. In this chapter, we discuss a selection of these diseases, the identified genes, and the underlying mechanisms causative for either decreased bone resorption or increased bone formation.

2 CLINICAL ASPECTS OF THE SCLEROSING BONE DISORDERS

In their most recent report, the working group on the Nosology and Classification of Genetic Skeletal Disorders reports more than 40 different clinical entities characterized by an increased bone density.[1] The fact that these are divided into three different groups indicates the heterogeneity among these conditions. In Tables 29.1 and 29.2, the most important radiological and clinical aspects of some of the most common forms of these diseases are summarized and classified based on their underlying pathogenic mechanisms. The latter is a key determinant for fracture risk in these patients. Intuitively, one would expect a decreased fracture risk as a consequence of increased bone mass but, as will be described later, the bone tissue present could be of inferior quality due to a decreased bone resorption or increased bone turnover, leading to an increased fragility of the bone. When there is increased bone formation, the bone tissue might be of perfect structure and result in bones with increased strength.[2]

Many sequellae can occur secondary to the increased bone mass. These complications can include anemia caused by obliteration or narrowing of marrow spaces of the long tubular bones or cranial nerve palsies from narrowed foramina in the skull. In several conditions, encroachments on the cranial nerves occur, causing hearing loss, optic nerve atrophy, and facial nerve palsy. Dental problems, osteomyelitis of the mandible, as well as osteoarthritis can be additional problems.[2] In the past, a complete clinical and radiological evaluation was needed to make an exact diagnosis within this group of rare conditions, but even then such a diagnosis was not always easy to make because of the overlap between several of the sclerosing bone disorders.[2]

3 MOLECULAR GENETICS AND PATHOGENIC MECHANISMS

3.1 Disorders Caused by Bone Resorption Defects

3.1.1 Osteopetroses

Osteoclasts, multinucleated cells of hematopoietic origin, are responsible for the resorption of bone.[35] The rate at which these cells resorb bone is determined both at the level of differentiation (osteoclast number) and through the regulation and functioning of key proteins with specific activities within the resorbing osteoclast.[36] The osteopetroses are a heterogeneous group of sclerosing bone dysplasias, marked by the inability of osteoclasts to resorb bone due to defects in the osteoclastogenesis

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TABLE 29.1 Principle Genetic Disorders Characterized by High Bone Mass (HBM) From Impaired Bone Resorption

Diseases	Forms	Genes	Mutation types	Functions	Major radiographic features	References
Osteopetrosis	D (X-linked)	*NEMO*	Hypomorphic	Oc differentation	Osteopetrosis is variable	3
	ARO	*TNFSF11*	Loss of function	Oc differentiation	Generalized increased bone density, sclerotic cranial base, metaphyseal clubbing (Erlenmeyer flask deformity), loss of trabecular structure, poor definition between cortical and medullary bone	4
		TNFSF11A				5
		CAII		Acidification by Ocs		6
		TCIRG1				7
		CLCN7				8
		OSTM				9,10
		SNX10				11,12
	IARO	*PLEKHM1*	Loss of function	Acidification by Ocs	Generalized sclerosis, widened metaphyses	13
		CLCN7	Partial loss of function			
	ADO	*CLCN7*	Dominant negative effect	Acidification by Ocs	Generalized sclerosis predominantly at vertebral endplates, iliac wings, and skull base	14
Pycnodysostosis	AR	*CTSK*	Loss of function	Collagenase activity	Short stature, dolichocephaly, open fontanelle, clavicular dysplasia, obtuse angle of the mandible, short terminal phalanges, generalized sclerosis	15
Osteosclerotic metaphyseal dysplasia	AR	*LRRK1*	Loss of function	Reduced bone resorption capacity	Severe osteosclerosis localized to the bone ends, wide sclerotic bands in the metaphysis, and the vertebral endplates are osteosclerotic	

All genes were identified through positional cloning, except for *CAII* (biochemical analysis) and *TNFSF11*(*A*) (both candidate gene approach).
A, Autosomal; AD, autosomal dominant; ADO, autosomal dominant osteopetrosis; AR; autosomal recessive; ARO, autosomal recessive osteopetrosis; D, dominant; IARO, intermediate autosomal recessive osteopetrosis; Oc, osteoclast; R, recessive.

or the acidification of the extracellular compartment.[37–39] The different forms of osteopetrosis in humans, as well as the many knockout or transgenic mouse models showing osteopetrosis, have been instrumental in our current understandings of the mechanisms of bone resorption.

The histological hallmark of the osteopetroses is remnants of unresorbed cartilage in the mature bone, which also explains their reduced strength.[40] The different individual forms of osteopetrosis are traditionally classified on the basis of inheritance, age of onset, severity, secondary clinical features, and more recently the number of osteoclasts.[37] However, the identification of the primary molecular genetic defect is now of greater relevance for the prognosis and treatment of the patient.[37]

A small subset of cases are characterized by a reduced number of osteoclasts, indicating an impaired osteoclast differentiation.[41] They are called the osteoclast-poor osteopetroses.[41] X-linked osteopetrosis, anhydrotic ectodermal dysplasia, and immunodeficiency (OLEDAID) is caused by hypomorphic mutations in the nuclear factor-kappa B (NFκB) essential modulator (NEMO),[3] an upstream signaling protein of NFκB, which is a key regulator of osteoclastogenesis. The affected boys show a broad spectrum of phenotypes; all suffer from immunodeficiency, and most of them have anhydrotic ectodermal dysplasia, whereas osteopetrosis, lymphedema, and hemangioma are less-frequent complications.[3] The mutations disrupt, but do not abolish, the function of NEMO.

Sobacchi et al. identified mutations in the *TNFSF11* gene encoding the receptor activator of nuclear factor kappa B (RANK) ligand (RANKL) as the cause of severe autosomal recessive osteopetrosis (ARO) in six patients whose bone biopsy specimens lacked osteoclasts.[4] In comparison with the more common osteoclast-rich forms of "malignant" or severe osteopetrosis, the disease progression in these patients is slower. Due to absence of a microenvironment enabling osteoclastogenesis, hematopoietic stem cell transplantation would probably not improve bone remodeling.[41] These patients might benefit from RANKL administration early in the disease course, or bone marrow mesenchymal stem cells transplantation

TABLE 29.2 Principle Genetic Disorders Characterized by HBM From Enhanced Bone Formation

Diseases	Forms	Genes	Mutation types	Functions	Major radiographic features	References
HBM	AD	*LRP5*	Gain of function	Wnt signaling coreceptor	Dense bones and cortical hyperostosis mainly affecting cranial/tubular bones	16,17
van Buchem	AR	*SOST*	52-kb deletion downstream	Wnt signaling suppressor	Hyperostosis of the calvaria, the skull base, and the mandible	18
Sclerosteosis	AR	*SOST*	Impaired or loss of function	Wnt signaling suppressor	Hyperostosis of the calvaria, the skull base, and the mandible; syndactyly; tall stature	19,20
	AR	*LRP4*	Loss of function			21,22
OSCS	D (X-linked)	*WTX*	Loss of function	Wnt signaling suppressor	Metaphyseal striations, sclerosis of the base of the skull, absent fibulae	23
Osteopoikilosis	AD	*LEMD3*	Loss of function	TGFβ and BMP signaling suppressor	Osteosclerotic foci, often asymptomatic, sometimes skin lesions	24
CMD	AD	*ANK*	Still unknown	Ob differentiation	Metaphyseal flaring, hyperostosis, and sclerosis of the cranial bones	25,26
	AR	*GJA1*	Unknown	Intracellular communication	Widening of the phalanges and flaring and undertrabeculation of the diaphysis of the long bones	27
CED	AD	*TGFβ1*	Gain of function	Ob differentiation/ proliferation	Cortical thickening and sclerosis of diaphyses of the long bones by both endosteal and periosteal proliferation, sclerosis of the basilar portions of the skull	28,29
JML	AR	*PTDSS1*	Gain of function	PS synthesis	Hyperostosis of the cranium, vertebrae, and the diaphysis of the long bones	30
TDO	AD	*DLX3*	Loss of function	DNA binding and regulation of gene expression	Enamel with less mineralization and thickening of the cortical bones, mandibular prognathism	31,32
Raine syndrome	AR	*FAM20C*	Loss of function	Upstream regulator of FGF23, role in biomineralization	Generalized increased bone density with marked increase in the ossification of the skull	33,34

AD, Autosomal dominant; D, dominant; AR, autosomal recessive; BMP, bone morphogenetic protein; CED, Camurati–Engelmann disease; CMD, craniometaphyseal dysplasia; Ob, osteoblast; OSCS, osteopathia striata with cranial sclerosis; PS, phosphatidylserine; TDO, Tricho–dento–osseous; TGFβ, transforming growth factor beta.

to replete cells that produce RANKL and thereby restore osteoclastogenesis, but so far none of these treatments have been reported. Most recently, mutations in the *TNFRSF11A* gene encoding RANK were found to underlie severe ARO in eight patients from seven unrelated families.[5] The patients suffered from increased bone density, anemia, severe narrowing of the optical foramina, and hypogammaglobulinemia and three died at a young age. Human stem cell transplantation represents a possible cure in this type of ARO.

The fact that these three genetic diseases and their underlying defects result in a reduced number of osteoclasts is not unexpected because of the essential role of the NFκB pathway in osteoclastogenesis. Another essential cytokine for osteoclast differentiation is macrophage colony-stimulating factor (M-CSF).[42–44] This cytokine is able to induce RANK expression in osteoclasts, priming these cells to differentiate in response to RANKL.[45] The cross talk between osteoblasts/stromal/activated T cells and osteoclast precursors is essential for correct osteoclast differentiation, as these cells produce RANKL and its decoy receptor, osteoprotegerin (OPG).[45–50] The ratio between OPG and RANKL that impacts the process is an important regulator for osteoclastogenesis both in vivo and in vitro. Binding of both M-CSF and RANKL is essential for the commitment of precursor cells to differentiate into mature osteoclasts.[51] Binding of RANKL to RANK leads to the intracellular recruitment of adaptor molecules, such as TNF receptor–associated cytoplasmic factor 6 (TRAF6), which in turn leads to the activation of NFκB and several mitogen-activated kinases (p38, Erk, and JNK).[50,52–54]

Immunoreceptor tyrosine-based activation motif (ITAM)–containing proteins, such as Fc receptor common γ subunit (FcR γ) and DNAX-activating protein 12 (DAP12), associate with immunoreceptors.[55–57] These

immunoregulatory adaptor molecules are essential for osteoclastogenesis, as mice lacking either protein lack osteoclasts.[55–57] Activation of the RANKL/RANK axis and ITAM-signaling induces nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), a key regulator of osteoclast differentiation, that leads to osteoclast differentiation and functional resorption of bone[58] (Fig. 29.1).

Other forms of human osteopetrosis have a normal or even high number of osteoclasts, suggesting normal differentiation but reduced bone resorption due to an impaired osteoclast functioning. In the first form, severe ARO is associated with renal tubular acidosis. It has been described in less than 200 families, most of them originating from the Mediterranean region and the Middle East. Clinical manifestations include short stature, cerebral calcifications, mental retardation, dental malocclusion, and fractures.[59] Most of the patients have a combination of proximal and distal renal tubular acidosis. Usually, there are no hematological manifestations of the disease. Loss-of-function mutations in the carbonic anhydrase II (*CAII*) gene cause this form of osteopetrosis.[6]

Malignant or infantile ARO is the most severe form of osteopetrosis and has an incidence of 1:200,000–1:300,000.[37] The disease is early manifesting and typically diagnosed soon after birth. There is a homogenous increase in bone density with lack of corticomedullary differentiation and apparent loss of trabecular structure. Fractures are common due to brittle sclerotic bones.[39,60,61] The excess bone impairs hematopoiesis, which often leads to extramedullary hematopoiesis and hepatosplenomegaly. This genetic form of osteopetrosis is often associated with neurological impairment due to narrowing of the foramina of the skull base.[41] If untreated, children usually die in their first decade as a consequence of recurrent infections.[41] Defects in three genes are known to impair osteoclast functioning and thereby cause malignant ARO. The majority of mutations (~50%) thus far are located in the *TCIRG1* gene encoding a subunit of the vacuolar H^+-ATPase (V-ATPase) proton pump that transports the generated protons across the osteoclast ruffled border into the resorption lacuna.[7] Less common are loss-of-function mutations in chloride voltage-gated channel 1, *CLCN7*, the gene that encodes a chloride channel required for maintenance of cell electroneutrality, and osteopetrosis-associated transmembrane protein 1 (*OSTM1*) that serves as a β-subunit for CLCN7 (Fig. 29.1).[8–10,14] In contrast to the osteopetrosis patients with mutations in *TCIRG1*, these patients suffer from retinal atrophy and neurodegeneration.[41] Also, hematopoietic stem cell transplantation will not fully cure these cases because this procedure has no effect on the neural system.[41] Currently however, not all malignant ARO cases can be explained by mutations in any of these three genes that regulate acid secretion by osteoclasts, highlighting the existence of at least one other gene causing this severe form of osteopetrosis.[62] The pathogenic mechanisms caused by mutations in all the genes found in these three autosomal recessive forms of osteopetrosis is shared among them. They all participate in the acidification of the extracellular compartment between the osteoclast and the bone tissue, which is essential for resorption of the extracellular matrix. To first dissolve the mineral matrix of bone, osteoclasts secrete large amounts of protons, which are formed by CAII.[6–10,14,60–63] V-ATPase transports the formed protons across the ruffled border into the resorption lacuna.[7,36,51] Bicarbonate/chloride transporters (EA proteins) in the plasma membrane transport the excess of formed bicarbonate out the cell.[64] Chloride channels, of which CLCN7 is likely to be the most important in the bone, ensure electroneutrality.[8] CLCN7 requires another protein, OSTM1, as a β-subunit for its stability.[65,66] When no acidic environment is created, the mineral matrix of the bone tissue cannot be dissolved, consequently the organic matrix cannot be degraded by proteolytic enzymes.

In a few consanguineous families of Palestinian origin a homozygous missense mutation (Arg51Gln) is present in the *Sortin Nexin 10* (*SNX10*) gene.[11] The patients showed hepatosplenomegaly, macrocephaly, and failure to thrive but normal neurocognitive development. Hearing was normal but functional vision was impaired due to severe optic atrophy. Bone marrow transplantation was successful is some patients. Later on, Pangrazio et al. reported the identification of 9 novel mutations in *SNX10* in 14 ARO patients from 12 unrelated families. These included three cases of "Västerbottenian osteopetrosis," named for the Swedish Province where a higher incidence of the disease has been reported.[12] The pathogenesis of this form of osteopetrosis is likely linked to the fact that SNX10 was shown to coimmunoprecipitate with V-ATPase and regulate its intracellular trafficking.[67] Autosomal dominant osteopetrosis (ADO) or Albers–Schönberg disease has a prevalence estimated at 5.5:100 000.[68] Onset of clinical and radiological manifestations usually occurs in late childhood or adolescence, although earlier onset has been reported.[38] These patients display a generalized osteosclerosis, predominantly at the vertebral endplates (Rugger–Jersey spine), the iliac wings, and the skull base.[2] Patients with ADO have also an increased fracture risk.[68] The most frequently fractured bone is the femur, but fractures in any long tubular bone and in the arch of the vertebrae occur.[69] Hip osteoarthritis and lumbar scoliosis may develop in adulthood.[69] Other clinical features are osteomyelitis of the mandible associated with dental carries or abscesses, anemia with extramedullary hematopoiesis, and cranial nerve involvement, but neurological signs are rather uncommon.[69] Most cases of ADO have been associated with heterozygous missense mutations causing a dominant negative effect on the homodimeric CLCN7 protein.[14,70,71]

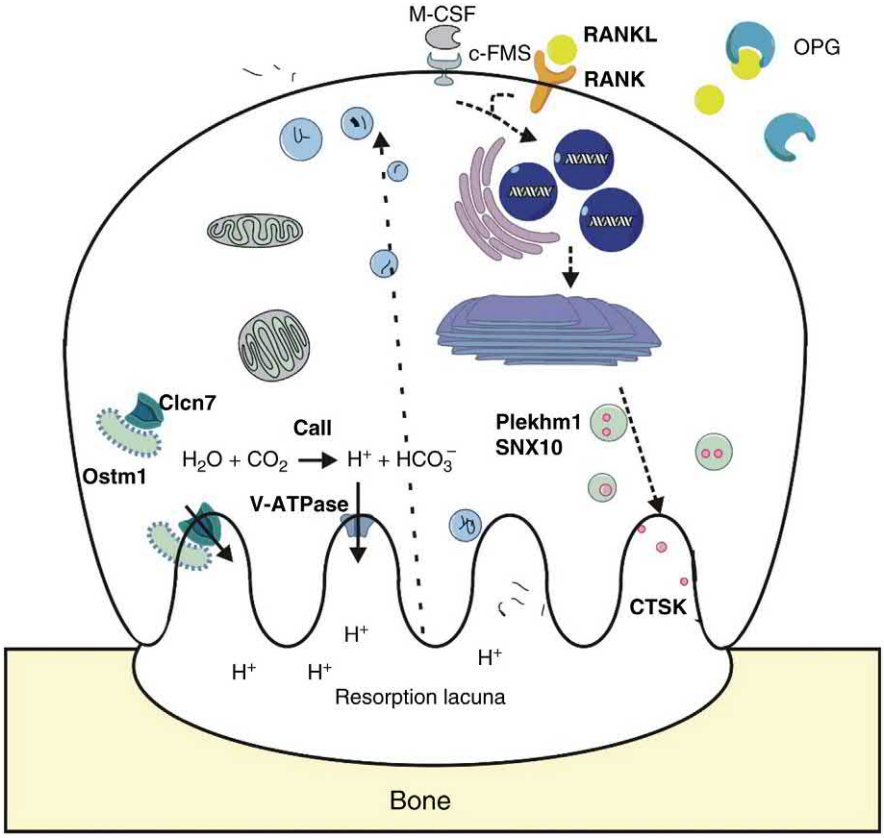

FIGURE 29.1 Osteoclast functioning. The molecular architecture of bone resorption by osteoclasts, that is, RANKL and macrophage colony-stimulating factor (M-CSF) binding to their receptors, induces the transcription of specific genes essential for osteoclastogenesis. In turn, the extracellular osteoprotegerin (OPG)/RANKL ratio is an important factor for osteoclast differentiation. Proteolytic enzymes and protons are secreted by active osteoclasts peripherally into the resorption lacuna. The resulting "debris" is centrally endocytosed at the ruffled border, and then secreted at the basolateral side of the osteoclast. Genes shown to be involved in human osteopetrosis and pycnodysostosis are indicated in bold. *CAII*, Carbonic anhydrase II; *c-FMS*, colony-stimulating factor 1 receptor; *Clcn7*, chloride voltage-gated channel 1; *CTSK*, cathepsin K; *Plekhm1*, pleckstrin homology domain containing, family M (with RUN domain) member 1; *Ostm1*, osteopetrosis-associated transmembrane protein 1; *SNX10*, Sortin Nexin 10; *V-ATPase*, vacuolar H^+-ATPase.

Patients with intermediate autosomal recessive osteopetrosis (IARO) display the typical radiological features of osteopetrosis, including generalized osteosclerosis and widened metaphyses.[37] The disease may be associated with short stature, dental problems, osteomyelitis, and high fracture risk.[37] IARO can be differentiated from the malignant form because the course is less severe, and life time expectancy is greater. Defects in two genes are known to cause this form of osteopetrosis: *CLCN7*[70] and pleckstrin homology domain containing, family M (with RUN domain) member 1 (*PLEKHM1*).[13] It is believed that CLCN7, being a homodimeric transporter, results in ADO when there is coupling of normal and mutant CLCN7 dimers, the latter apparently inefficient in Cl^- transport.[68] As discussed earlier, homozygosity or compound heterozygosity for loss-of-function mutation of *CLCN7* results in ARO, whereas IARO is due to missense mutations that only mildly reduce Cl^- conductance.[72]

Finally, mutations in the *PLEKHM1* gene underlie an intermediate type of human osteopetrosis in two siblings.[13] The clinical outcome in this family was mild. The oldest patient has Erlenmeyer flask deformities of the distal femora and chondrolysis of a hip. Her younger brother does not have clinical features. The function of the PLEKHM1 protein is not precisely known, but it has been suggested to be involved in osteoclast vesicular transport.[13] The ruffled border, a highly infolded plasma membrane, is believed to form by fusion of many acidic vesicles with the peripheral region of the bone-facing plasma membrane.[73] In the second step of bone resorption, the organic component is dissolved by the secretion of a number of enzymes, including the proteolytic enzyme cathepsin K, tartrate-resistant acid phosphatase, and matrix metalloproteinases into the resorption lacuna. Matrix degradation products and debris are endocytosed from the central portion of the ruffled border and packaged into transcytotic vesicles. These are

transported throughout the cell, and secreted at the basolateral side of the osteoclast[74] (Fig. 29.1). The PLEKHM1 protein could play a role in any of these processes.

The physiological role of most of the genes involved in the differentiation or functioning of osteoclasts was confirmed in animal models. Either spontaneous or induced transgenic mice for most of these genes show different forms of osteopetrosis, as reviewed by Van Wesenbeeck and Van Hul.[75] When considering human forms of osteopetrosis, it is remarkable that defects of only a small subset of these genes are involved. A possible explanation, is that several of the genes mentioned have a pleiotropic role, and mutations in these genes might be lethal in humans. On the other hand, in a subset of cases with ARO and IARO, the underlying genetic defect is still unknown.[41] Therefore, involvement in these unresolved cases of some of the genes mentioned cannot yet be excluded.

3.1.2 Pycnodysostosis

Pycnodysostosis is a rare autosomal recessive disorder due to an impaired degradation of the organic matrix of bone by osteoclasts.[76] The main characteristics are short stature, dolichocephaly, open fontanelle, clavicular dysplasia, obtuse angle of mandible, short terminal phalanges, and general increased bone density and fragility.[77] Pycnodysostosis is caused by loss-of-function mutations of the *Cathepsin K* (*CTSK*) gene.[15,78] Twelve different mutations, spread throughout the gene are described.[78] CTSK is responsible for the degradation of collagen type 1 at low pH[79–82] (Fig. 29.1). The osteoclasts of patients are polarized correctly and able to demineralize the matrix underneath the ruffled border.[83] Collagen degradation, however, does not occur, and these cells contain many cytoplasmic vacuoles filled with undegraded collagen.

3.1.3 Osteosclerotic Metaphyseal Dysplasia

Osteosclerotic metaphyseal dysplasia is an autosomal recessive disease characterized by severe osteosclerosis localized to the bone ends.[84] The clinical features include developmental delay, hypotonia, and seizures. Radiologically the metaphysis shows wide sclerotic bands and the vertebral end plates are osteosclerotic. The skull, however, remains unaffected. In one patient, a homozygous loss-of-function mutation was identified in the *LRRK1* gene.[85] Functional studies illustrated that the mutation reduced the bone resorption capacity of osteoclasts. An absence of a mutation in a few other patients suggests the genetic heterogeneity of this condition.[85]

3.2 Disorders Caused by Increased Bone Formation

Osteoblasts, cells of mesenchymal origin, are the bone-forming cells that synthesize and mineralize the skeleton. Osteoblast differentiation at the early stages is controlled by two important transcription factors: *Runx2* and *Osterix*. Mice deficient for either *Runx2* or *Osterix* do not show mature osteoblasts or calcified bones.[86–88] Additionally other transcription factors play a critical role in bone formation.[89] Major breakthroughs in the unraveling of the sclerosing bone dysplasias caused by an increased bone formation contributed greatly to our current understandings of skeletal biology. These findings have received considerable attention, especially because of potential therapeutic applications for anabolic treatment of osteoporosis.[90] More precisely, the role of the canonical Wingless and Int-1 (Wnt) signaling pathway in several of these disorders has impacted the field significantly.[91]

3.2.1 High–Bone Mass Phenotype, Sclerosteosis, and van Buchem's Disease

Positional cloning in two unrelated kindred in which a high–bone density phenotype (HBM) segregated as an autosomal dominant trait, revealed a gain-of-function mutation in the *low-density lipoprotein receptor–related protein 5* (*LRP5*) gene, a coreceptor for Wnt proteins (Fig. 29.2). The reported clinical findings range from asymptomatic to square jaw phenotype and torus lesions, even within one family, to cranial nerve compressions and otopharyngeal exostoses.[16,17] The role of this protein in bone formation was also revealed when loss-of-function mutations in this gene were found to cause osteoporosis pseudoglioma syndrome, featuring blindness and significantly very low bone mass at a young age.[92] The first mutation identified in the high–bone mass cases was a missense mutation in the first propeller domain, which has an activating effect on canonical Wnt signaling.[93] In the following years, patients before being (sometimes mis)diagnosed with endosteal hyperostosis, Worth disease, van Buchem disease, osteosclerosis, or ADO type I were also shown to carry a similar heterozygous, gain-of-function mutation in LRP5.[94] All mutations in *LRP5* clustered in the first β-propeller domain, situated in the extracellular part of the protein.[94] The bone phenotype, with dense bones and cortical hyperostosis mainly affecting the cranial and tubular bones, is similar in all patients.[94]

Without doubt, this evidence that the Wnt/β-catenin signaling pathway plays an important role in bone anabolics is the most significant breakthrough generated by studying sclerosing bone dysplasias.[91] The WNT proteins are a family of highly conserved, secreted, signaling molecules that regulate cell-to-cell interactions during embryogenesis.[95] In mammals, 19 such growth factors are described.[95] Porcupine (PORCN), an endoplasmic reticulum protein, is involved in the secretion and the lipidification of WNT proteins.[96,97] The Wnt pathway is induced by binding of a WNT molecule to its receptor complex; a member of the frizzled receptor (FZD) family, combined with a coreceptor, being either LRP5

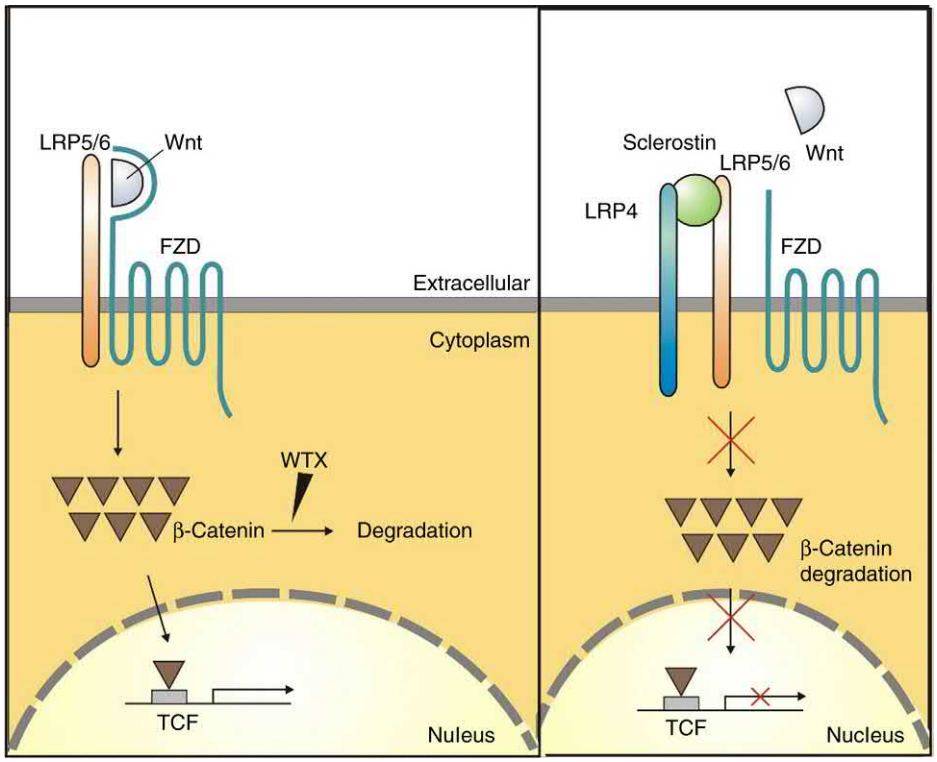

FIGURE 29.2 Schematic overview of the low-density lipoprotein receptor–related protein 5 (LRP5) osteoblast-dependent pathway that regulates bone formation. The direct stimulatory effect of LRP5 on bone formation involves the binding of Wingless and Int-1 (Wnt) ligands to the LRP5/6 and frizzled receptors (FZD) on the osteoblast, consequently inhibiting β-catenin degradation. Accumulation of β-catenin in the cytoplasm will eventually result in translocation to the nucleus where activation of target genes is initiated. In the presence of the LRP4–sclerostin inhibitory complex, the binding of Wnt ligands to the frizzled (FZD)–LRP5 receptor complex is prevented, which results in the degradation of β-catenin. *TCF*, T cell–specific transcription factor; *WTX*, Wilms tumor on the X-chromosome.

or LRP6.[95] Three signaling pathways are described: the canonical pathway (Wnt/β-catenin), and two noncanonical pathways (Wnt/Ca^{2+} and Wnt/PCP).[98] The activation of the best understood Wnt signaling pathway, the Wnt/β-catenin pathway, leads to the translocation of β-catenin to the nucleus where it associates with the lymphoid enhancer–binding factor (Lef)/T cell–specific transcription factors (Tcfs), leading to the transcription of target genes (Fig. 29.2).[99,100] The precise mechanism by which Wnt signaling increases bone formation seems to involve different levels. Canonical Wnt signaling promotes osteoblast differentiation from mesenchymal stem cells, while it inhibits both chondrocyte and adipocyte differentiation.[101] Furthermore, Wnt signaling enhances osteoblast proliferation and activation (mineralization) and suppresses osteoblast apoptosis.[102] This pathway also interferes with osteoclastogenesis by increasing the OPG/RANKL ratio.[103,104]

Sclerosteosis and van Buchem disease are two closely related bone diseases marked by progressive cortical thickening and generalized osteosclerosis. Sclerosteosis is considered to be an autosomal recessive trait, although radiological examination of several heterozygotes suggests an autosomal partial dominant inheritance.[105] Patients show hyperostosis of the calvarium, the skull base, and the mandible, resulting in compression of the cranial nerves and consequent facial palsy, hearing loss, optic neuropathy, loss of smell, and increased intracranial pressure.[105] This hyperostosis leads to characteristic facial features, such as a broad, squared mandible, hypertelorism, a flat nasal bridge, frontal prominence, and proptosis in several cases.[105] At birth, syndactyly of mostly the index and middle finger is present in a large percentage of patients. Also, tall stature is common in sclerosteosis.[19,106] van Buchem disease, first described in 1955,[107] is marked by a similar but milder phenotype than sclerosteosis. Also, syndactyly is not present in patients.[107] Genetic studies showed that both disorders are allelic, and linked to a genetic defect in the *SOST* gene coding for sclerostin.[19,20,108,109] So far, different loss-of-function mutations in *SOST* are reported in patients with sclerosteosis.[19,20] A few years ago, we reported the first missense mutation in this gene, which mutated a cysteine residue and resulted in a sclerosteosis phenotype.[110] In van Buchem patients, others and we have shown that a 52-kb deletion downstream of the *SOST* gene suppresses its expression, thus causing a similar phenotype.[18]

Due to the presence of a cysteine knot motif in the sclerostin protein and homology of the *SOST* gene with members of the gremlin gene family, sclerostin was initially suggested to be a bone morphogenetic protein (BMP) inhibitor. However, it is a Wnt inhibitor.[106] Due to its pleiotrophic role, the regulation of Wnt signaling is very important and occurs at different levels. At first, extracellular regulation is performed by several proteins. It is well established that the coreceptors LRP5 and LRP6 have a central role in this metabolic pathway and its regulation.[92] The discoveries that both activating and loss-of-function mutations in *LRP5* have dramatic effects on bone density indicated the role of the Wnt pathway in bone homeostasis.[16,17,92] This was subsequently supported by the fact that inhibitory effects of sclerostin are absent in sclerosteosis and van Buchem disease, and that sclerostin is a Wnt inhibitor rather than a BMP inhibitor. The mode of sclerostin action turned out to be the binding to LRP5, thus inhibiting the binding of the Wnt ligand to its receptor complex.[106] Furthermore, others and we showed that the activating missense LRP5 mutations that cause HBM disrupt the binding capacity with sclerostin,[111–113] explaining the radiological and clinical similarities between patients with HBM phenotype and those with sclerosteosis/van Buchem disease. Recently, we obtained further support for this pathogenic mechanism by the identification of three missense mutations in *LRP4* in patients with a sclerosteosis phenotype.[21,22] LRP4 can regulate the Wnt/β-catenin signaling pathway activity and consequently bone formation by binding to sclerostin and facilitating the inhibitory action of sclerostin. All three sclerosteosis-causing mutations in LRP4 are located in the cavity of the third β-propeller domain of the protein and functional studies demonstrated that they disrupt the binding with sclerostin.[21,22] Due to this reduced binding the Wnt-inhibitory capacities of sclerostin are decreased and recent studies in human and mice demonstrated that loss of LRP4 function in the osteoblasts results in increased serum levels of sclerostin.[21,114] This suggests that LRP4 has a role in sequestering sclerostin at the bone surface and making it available for the binding with LRP5/6 and the inhibition of the canonical Wnt pathway. Besides sclerostin, Dickkopf (DKK) proteins are also secreted regulators of Wnt, which can bind to the coreceptor LRP5/6 and prevent the formation of the LRP—FZD-Wnt complex, thus inhibiting the induction of Wnt signaling.[115] Furthermore, in the presence of kremen1 or kremen2 (krm1/2), two transmembrane proteins, a tertiary complex between krm, DKK, and LRP5/6 can be formed, which can be internalized, thus reducing the availability of this coreceptor for its Wnt ligands.[116] Alternative extracellular mechanisms to inhibit Wnt signaling involve members of the secreted frizzled-related proteins (sFRPs), which have domains that can bind to WNTs and thus prevent the binding of the latter to their receptor complex.[117] In a similar way, Wnt inhibitory factor-1 (Wif-1) can bind to WNTs, thereby suppressing Wnt signaling.[118]

In the cytosol, as well as the nucleus, additional mechanisms are available for regulating the canonical Wnt signaling pathway.[100] APC, AXIN, and Wilms tumor on the X-chromosome (WTX) form (together with other factors) the β-catenin "destruction complex." This complex facilitates the phosphorylation of β-catenin by casein kinase 1 (CK1). This leads to the ubiquitination and proteosomal degradation of β-catenin and negatively regulates the Wnt/β-catenin signaling pathway.[100] Finally, the nuclear availability of transcription factors TCF and LEF, and cofactors can influence the signaling.[100]

In 2008, Yadav et al. reported evidence that LRP5 can also act in a β-catenin–independent manner[119] by inhibiting serotonin synthesis in enterochromaffin cells in the duodenum. Gut, but not osteoblast-specific, LRP5 appeared to regulate the expression of tryptophan hydroxylase 1 (*Tph1*), the rate-limiting biosynthetic enzyme for serotonin production in enterochromaffin cells. High peripheral, gut-derived serotonin levels inhibit osteoblast proliferation through the 5-HT receptor (Htr1b) on the osteoblasts.[119,120] These discoveries conflict with the common belief that the function of LRP5 on bone resides in the osteoblast itself. To explain the discrepancies between the two hypotheses, Cui et al.[121] generated several mouse models and reported no effect on bone mass when they expressed *Lrp5* HBM alleles or inactivated WT *Lrp5* in gut cells, while they did observe a HBM phenotype or a decrease in bone mass when the expression was targeted to osteocytes and late osteoblasts, respectively. Furthermore, no support could be found for *Lrp5* genotype differences in the amount of serum serotonin.[121] They concluded that there is a local effect of the Wnt/β-catenin pathway in osteocytes and late-stage osteoblasts, rather than regulation of serotonin production by LRP5 in the gut.[121] So far, additional studies have not yet been able to explain the discrepancies between these two sets of different studies.

3.2.2 *Osteopathia Striata With Cranial Sclerosis*

Osteopathia striata with cranial sclerosis (OSCS) is a sclerosing bone dysplasia that presents in females with macrocephaly, cleft palate, mild learning disabilities, sclerosis of the long bones and skull, and longitudinal striations visible on radiographs of the long bones, pelvis, and scapulae.[122,123] It is an X-linked dominant disease, but in hemizygous males this entity is usually associated with fetal or neonatal lethality due to severe heart defects and/or gastrointestinal malformations.[23,122–124] It is often accompanied by bilateral fibula aplasia. Jenkins et al., identified mutations in the gene encoding WTX, a repressor for Wnt signaling, as the cause of X-linked OSCS.[23] Rivera et al. showed that WTX, besides

its functioning in Wnt signaling, might also play a role in nuclear pathways implicated in the transcriptional regulation of cellular differentiation programs.[125] We also performed mutation analysis of a large set of families and patients and confirmed the causality of *WTX* mutations in all cases, and expanded the clinical spectrum of both affected females and males.[124]

WTX encodes a 1135–amino acid protein that contains an acidic domain and three APC-binding domains (APCBD1–3).[126] At the N-terminus, a phospholipid binding [PtdIns(4,5)P_2] activity is localized. A β-catenin–binding domain is localized at the C-terminal of Gly^{368}. *WTX* has two splice forms, WTX_{S1} and the shorter WTX_{S2} resulting from exclusion of residues 50–326. Both isoforms retain the ability to bind β-catenin, but only WTX_{S1} is localized to the plasma membrane, and is therefore important for the suppression of WNT signaling.[23] This seems to be illustrated by the fact that the presence of intact WTX_{S2} is not protective against the disease. The wide phenotypic spectrum and range of severity of OSCS cannot be explained solely by the type of *WTX* gene defect because there is a highly variable expression of the disease within one family. Some individuals are very mildly affected and even asymptomatic, whereas others have severe complications.[23,124] Nonrandom X-inactivation in OSCS could provide an explanation for the clinical variation seen in females. For example, unfavorable nonrandom X-inactivation could possibly worsen the prognosis. For males, Jenkins et al.[23] suggested a possible genotype–phenotype correlation that relates the position of the mutations in *WTX* with patient survival: only mutations that produce a WTX_{S1} with intact PtdIns (4,5)P_2 and APCBD1 domain result in survival. Our data indicated that this correlation is less clear.[124] The *WTX* gene defect identified in one male patient (p.Q271X) resulted in a highly truncated protein with an intact PtdIns(4,5)P_2 domain but without the APCBD1 domain. Offspring include two affected but surviving males, one being 20 years. It appears that one type of *WTX* mutation cannot be associated with a wide range of clinical features, presumably due to unknown modifying factors.[23,124] Isolated osteopathia striata has long been considered as a benign radiographic finding, marked by sclerosis of the bones and linear striations in the metaphyseal region of the long bones and pelvis. However, OSCS caused by *WTX* mutations should also be considered in cases of cranial sclerosis in a male patient because longitudinal striations are hardly ever seen in affected males with OSCS. Recently, a male patient with longitudinal striations was found to represent mosaicism upon DNA sequencing.[127] These cumulative findings suggest that a mixture of affected and nonaffected osteoblasts in the growth plate, due to random X-inactivation in females or mosaicism in males and females, is needed to generate longitudinal striations of the long bones. OSCS shares several clinical findings with focal dermal hypoplasia: cleft palate, short stature, and striations.[128] Not surprisingly, *PORCN*, an endoplasmic reticulum protein involved in secretion of WNT proteins, was identified by Wang et al. as the causative gene.[129]

3.2.3 *Osteopoikilosis and Melorheostosis*

Osteopoikilosis, literally meaning "spotted bones," is a usually benign condition characterized by osteosclerotic foci that occur in the epimetaphyseal regions of long bones.[130] These findings are usually noted in the shoulders, wrists, knees, ankles, pelvis, and scapulas. Radiographically, the hyperostotic spots in osteopoikilosis show symmetric but unequal distribution in different parts of the skeleton.[130] They represent loci of old remodeled bone with lamellar structure, either connected to adjacent trabeculae of spongy bone or attached to the subchondral cortex.[131]

Osteopoikilosis is usually found incidentally because patients are often asymptomatic. Sometimes, however, it is associated with skin manifestations, as Buschke–Ollendorff syndrome (BOS).[132] The skin lesions represent disseminated connective tissue nevi with both elastic-type nevi (juvenile elastoma) and collagen-type nevi (dermatofibrosis lenticularis disseminata) being described.[24]

In a few patients with osteopoikilosis, melorheostosis can be present.[133] The latter condition is characterized by linear hyperostosis of the cortex of long bones reminiscent of dripping candle wax (*rheos* means flowing in Greek). In sporadic cases of melorheostosis, these bony lesions are often accompanied by abnormalities of adjacent soft tissues, such as joint contractures, sclerodermatous skin lesions, muscle atrophy, hemangiomas, and lymphedema.[134] In melorheostosis, the lesions are usually asymmetric, they may involve only one limb or correspond to a particular sclerotome.[134]

Osteopoikilosis and BOS segregate in an autosomal dominant manner with considerable intrafamilial variation. Hellemans et al. identified heterozygous loss-of-function mutations in the gene encoding LEMD3.[24] LEMD3 is an integral nuclear membrane protein that can inhibit BMP and transforming growth factor beta (TGFβ) signaling.[24]

Melorheostosis is mostly encountered as an isolated, sporadic disorder. However, it seems to have increased prevalence in families with osteopoikilosis or BOS.[129] It was hypothesized that a second somatic mutation in the LEMD3 gene may cause sporadic melorheostosis. However, no experimental evidence to support this hypothesis has been obtained so far.[24] Hellemans et al. took skin biopsy samples from two affected individuals, one from an elastic-type nevus in a person with BOS, and the second from a hard scleroderma-like lesion in an individual with melorheostosis and BOS, but no somatic mutation (second hits) in the LEMD3 gene was identified.[24]

3.2.4 Craniometaphyseal Dysplasia

Craniometaphyseal dysplasia (CMD) is marked by abnormal modeling of the long bones, resulting in metaphyseal flaring, together with hyperostosis and sclerosis of the cranial bones.[135] The facial abnormalities include a thick bony wedge over the bridge of the nose and glabella and are usually the first sign of the disease. Other common clinical findings are impaired vision, hearing loss, and facial nerve paralysis.[135] A severe autosomal recessive form occurs,[136] but the autosomal dominant form is most common.[137] The dominant form of CMD is caused by mutations in the progressive ankylosis gene (*ANKH*), a transmembrane protein that transports intracellular pyrophosphate to the extracellular milieu.[25,26] Pyrophosphate is an inhibitor of mineralization and the *ANKH* mutations most likely decrease the protein's ability to transport pyrophosphate out of the cell.[25,26] Reduced extracellular levels of pyrophosphate increase bone mineralization, which may contribute to the bone overgrowth seen in CMD. Kirsch et al. showed a decreased expression of genes involved in osteoblast differentiation in the absence of functional ANKH, suggesting that ANKH is a positive regulator of osteoblast differentiation events toward a mature osteoblastic phenotype.[138] ANKH mutations are also associated with familial chrondrocalcinosis, which sometimes occurs in CMD.[139]

More recently, a homozygous mutation in the C-terminal region of the *gap junction protein alpha-1* (*GJA1*) gene was found in several families with the recessive form of CMD.[27] The patients with *GJA1* mutations have widening of the phalanges and flaring and undertrabeculation of the diaphysis of the long bones, which resembles the Erlenmeyer flask shape that is reported in patients with other metaphyseal dysplastic syndromes.[27] *GJA1* encodes connexin43 (Cx43), which plays a role in the formation of gap junctions that are important for intracellular communications. Cx43 is, of all connexins, the one that's most abundantly expressed in the bone.[140] Knockout of *Gja1* in mice results in delayed skeletal ossification, craniofacial abnormalities, and osteoblast dysfunction.[141,142] Furthermore, in vitro data also confirmed that Cx43 is important for the intracellular communication during osteoblast differentiation. Overall, studies have shown Cx43 has a role in the modulation of bone modeling and remodeling and that it is involved in the response to hormonal and mechanical stimuli by regulating the expression of osteoanabolic and -catabolic target genes.[140] However, the exact mechanism behind this regulation needs to be further elucidated.

3.2.5 Camurati–Engelmann Disease

Camurati–Engelmann disease (CED) or progressive diaphyseal dysplasia is a rare, autosomal dominant bone disorder marked by hyperostosis of the skull base, cortical bone thickening, and sclerosis of the diaphyses of the long tubular bones by both endosteal and periosteal bone proliferation.[143] Patients suffer from leg pain and muscular weakness.[143] CED is caused by domain-specific mutations in the *TGFβ1* gene,[28,29] which encodes a prepropeptide composed of the mature TGFβ1 and its latency-associated protein (LAP). LAP binds to mature TGFβ1 keeping it biologically inactive until its release. In health, this inactive complex is bound to the extracellular bone matrix through the latent TGFβ1-binding protein (LTBP). It has been suggested that the majority of the CED mutations, all localized in the LAP, weaken the bond between TGFβ1 and LAP, thereby promoting premature activation of TGFβ1 and subsequently increasing bone formation.[121] Once released, TGFβ1 can act as a ligand for a receptor complex on the cell surface, thus inducing TGFβ1 signaling through intracellular Smads.[144] Recently, it became clear that mature TGFβ1 is released during bone resorption, thereby inducing the migration of bone mesenchymal stem cells to the bone resorptive sites to initiate bone formation by osteoblasts, thus coupling bone resorption and formation.[145]

3.2.6 Lenz–Majewski Syndrome

Lenz–Majewski syndrome is a very rare, well-defined sclerosing bone dysplasia not only characterized by a progressive hyperostosis of the cranium, vertebrae, and the diaphysis of the long bones, but also marked by dwarfism, intellectual disability, and craniofacial, dental, cutaneous, and distal limb abnormalities.[146–150] Recently, Sousa et al. demonstrated that gain-of-function mutations in *phosphatidylserine synthase 1* (*PTDSS1*), encoding the phosphatidylserine synthase 1 (PSS1) are the cause of this rare disorder.[30] PSS1, together with PSS2, is responsible for the synthesis of phosphatidylserine (PS), which represents 3%–10% of all mammalian membrane phospholipids in tissues.[151,152] The presence of limb–mammary syndrome mutations results in increased synthesis of PS by the mutant PSS1 and a decreased negative feedback regulation of PSS1 activity by PS.[30] Although, no direct link has been described between PS and any of the pathways known to be involved in skeletal dysplasias, it is demonstrated that PS can bind calcium within matrix vesicles for beginning hydroxyapatite crystal formation and that it can enhance osteogenic differentiation of mesenchymal stem cells to promote bone formation.[30,153–155] To identify the exact mechanism whereby mutations in *PTDSS1* cause all different clinical features seen in the patients with limb–mammary syndrome, additional studies are needed.

3.2.7 Tricho–Dento–Osseous Syndrome

Tricho–dento–osseous (TDO) syndrome is a rare autosomal dominant disorder with a high penetrance and characterized by hair, tooth, and bone defects. The main

features are curly hair at birth, hypoplastic enamel with less mineralization, and thickening of the cortical bones. In addition, some patients also have brittle nails, mandibular prognathism, and taurodontism.[156–158] In 1998, the *distal-less homeobox 3* (*DLX3*) gene was identified as a disease-causing gene for TDO syndrome.[31,32] DLX3 contains three main domains, namely N- and C-terminal transactivation domains and a central homeodomain (amino acid 129–188), which can interact with DNA in a sequence-specific manner. In this way, DLX3 can regulate the expression of several target genes during a variety of developmental processes. So far, six different mutations have been described, all located in the homeobox domain.[159] In general, the missense mutations result in the most severe clinical phenotype; however, the severity of the clinical symptoms can also vary considerably between patients with the same mutation. This is probably the result of the genetic background of the patients and environmental factors.[159]

3.2.8 Raine Syndrome

Raine syndrome is a neonatal osteosclerotic bone dysplasia of early onset.[160] Most patients die within the first weeks of life, but some survive into childhood. Radiographically a generalized increase in the density of all bones is seen with a marked increase in the ossification of the skull. Characteristic facial features include narrow prominent forehead, depressed nasal bridge, proptosis, and midface hypoplasia. Periosteal bone formation is also characteristic, differentiating it from osteosclerotic bone dysplasias. In 2007, Simpson et al. identified loss-of-function mutation in the *FAM20C* gene encoding the dentin matrix 4 protein.[33] Later on some missense mutations were identified in patients with milder forms of the disease.[34] FAM20C is a kinase that phosphorylates several secreted proteins implicated in biomineralization.[161] It has been suggested to be an upstream regulator of the phosphate-regulating hormone fibroblast growth factor 23 (FGF23),[162] which could explain the biomineralization phenotype of Raine syndrome.

4 DIAGNOSTICS, TREATMENT, AND GENETIC COUNSELING

Identification of the underlying genetic defects for many of these sclerosing bone dysplasias has had several important implications for the patients and their families regarding the prognosis, genetic counseling, and even treatment of the disorders. However, all this starts with an exact diagnosis. Making this diagnosis has been proven to be very difficult in many cases. This can first be explained by the fact that for many of these conditions the number of cases reported in the literature is very few. Furthermore, there can be clinical and radiological overlap between many of these diseases.[2] Some cases even show a combination of features associated with different diagnoses, and are therefore categorized as so-called mixed sclerosing bone dysplasias or overlap syndromes.[2] Once a presumptive or "working" diagnosis is made, this should be confirmed by molecular analysis when recurrence risks or issues of treatment are under consideration. For some phenotypes, a major obstacle in achieving this is the genetic heterogeneity. For example, for the recessive cases of osteopetrosis, complete sequence analysis of several genes may be necessary to identify the genetic cause. Furthermore, in about 10%–15% of the osteopetrosis cases, none of the currently identified genes is involved, and novel gene identification(s) is awaited.[41] Now, with increased feasibility and applicability of next-generation sequencing technologies, these problems will most likely be solved by the use of specifically designed gene panels, by exome sequencing, or, in the near future, even whole-genome sequencing.

The identification of the precise molecular defect in osteopetrosis is also of relevance for prognosis, as well as treatment.[41] For example, when comparing the forms without any osteoclasts (i.e., osteoclast poor) with the forms with an increased number of, but functionally impaired, osteoclasts, somewhat unexpectedly, the former ones seem to be more mildly affected.[41] According to some investigators, this supports the hypothesis that osteoclasts secrete, currently still unidentified, anabolic factors that cause a exacerbating effect in osteopetroses patients who feature increased number of osteoclasts.[163] Another level of genotype–phenotype correlation can clearly be seen within the group of *CLCN7* mutants.[14] Complete loss of function causes a severe form, whereas reduction of the protein level to 50% has no effect on the functionality of the osteoclast, as seen in parents of ARO patients.[14] However, an intermediate phenotype is seen in ADO and IARO due to a heterozygous mutation with a dominant negative effect or by two mutations that only partially affect the functioning of the chloride channel, respectively.

Regarding treatment of the osteopetrosis, bone marrow transplantation has been applied with success on many occasions.[60] But again, this should only be considered when the defect is intrinsic to the osteoclast. Osteoclast-poor forms of osteopetrosis can only benefit from bone marrow transplantation when the defect is within the gene encoding the RANK protein, but no benefit can occur when the mutation is within the gene encoding RANKL.[41] Furthermore, one has to take into account that the neurological damage in the brain, seen in *CLCN7*-mutated cases, cannot be prevented by bone marrow transplantation.[41]

Regarding the treatment of sclerosing bone disorders in general, some attempts have included lowering bone mass using a low-calcium diet or by administering glucocorticoids.[164] As mentioned earlier, bone marrow

transplantation can cure some forms caused by an osteoclast intrinsic defect. However, for most cases treatment is focused on the symptoms secondary to the increased bone mass. For example, surgical decompression of narrowed cranial foramina is being performed to reduce cranial nerve palsies.[105] Also, cosmetic interventions to recontour the enlarged mandible, as well as surgical interventions to reduce the intracranial pressure, are being performed.[105]

As discussed, our understanding of the pathogenesis of sclerosing bone disorders has not always resulted in an effective treatments. However, despite the clinical complications, they definitely served as models for the development of therapeutic strategies to prevent and to treat osteoporosis. Today, antiresorptive therapy, that is, bisphosphonates and anti-RANKL antibody treatment, play important roles in the management of osteoporosis.[165] Similarly, efforts are ongoing to use some of the genes (*CTSK* and *CLCN7*) identified in conditions with impaired bone resorption as potential drug targets. However, antiresorptive therapy in general is expected to fail in the full restoration of the bone mass and quality of bone tissue already lost.[165] Therefore, the identification of genes involved in osteoblast differentiation, proliferation, and functioning are of major interest because these could lead to new agents that enhance bone formation, and consequently, restore bone structure.[166] Currently, several clinical trials with novel anabolic therapies are being conducted by many pharmaceutical companies, mainly focused on the inactivation of Wnt antagonists, such as DKKs, SFRPs, and sclerostin.[166] The latter target seems to be especially promising, as with its expression pattern being restricted to bone, side effects caused by interference with sclerostin function are likely to be minimal or absent.[167]

In conclusion, although many and significant breakthroughs have been realized, still a treasure of information remains hidden in the genetic disorders that increase bone mass. The functions of many genes and pathways are yet to be understood. Furthermore, not all genes responsible for these rare bone dysplasias have been identified and additional key factors in the processes of bone homeostasis are awaiting their unraveling.

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CHAPTER

30

Fibrodysplasia (Myositis) Ossificans Progressiva

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1 INTRODUCTION

Fibrodysplasia ossificans progressiva (FOP) is a human genetic disorder (MIM #135100; http://omim.org/entry/135100) in which bone forms episodically in soft connective tissues during childhood and throughout adult life, often in response to injury. Such extraskeletal bone formation, or heterotopic ossification (HO), is an extremely rare clinical finding in children, although HO in adults is frequently associated with a number of common conditions that involve severe trauma, such as spinal cord and head injuries, hip replacement surgery, and war-induced blast injuries.[1] Recent advances over the past several years, using high-fidelity mouse models of FOP, have led to a better understanding of the molecular and cellular consequences of the *ACVR1* gene mutations that cause this genetic disease. These tools continue to provide new knowledge about the mechanisms that induce HO and regulate bone formation, as well as insight into the treatment approaches for FOP and other disorders of cartilage and bone formation.

2 CLINICAL DESCRIPTION: FIBRODYSPLASIA OSSIFICANS PROGRESSIVA

Fibrodysplasia ossificans progressiva (FOP) is recognized by two characteristic clinical features: (1) progressive formation of extraskeletal bone, or HO, and (2) congenital malformations of the great toes (Fig. 30.1).

2.1 Heterotopic Endochondral Ossification in FOP

Children with FOP develop painful, highly inflammatory soft tissue swelling. Onset typically occurs within the 1st decade of life, although episodes as early as the first year after birth have been reported, as has initial onset occurring much later in life.[2–5] This swelling, known as a flare-up, appears suddenly, expands rapidly, and is highly vascular.[6] A flare-up is the first clinical indication of the soft connective tissue inflammation and degradation that precedes tissue replacement by extraskeletal bone through a process of cell death followed by endochondral ossification.[7–9] Patients report a variety of symptoms, including swelling, pain, stiffness, decreased movement, and warmth.[10] In some cases, a flare-up subsides without residual bone formation, but most cases result in HO and some loss of function.[10] Although skeletal muscle is the tissue most affected by HO, extraskeletal bone also forms in other soft connective tissues, including aponeuroses, fascia, ligaments, and tendons.[11] Heterotopic bone formation in FOP is progressive, cumulative, and severely disabling.[10]

Nonhereditary forms of HO arise in response to severe tissue trauma; in contrast, HO can be triggered in FOP patients by minor tissue injury, such as intramuscular immunizations, mandibular blocks for dental work, severe muscle fatigue, blunt muscle trauma from bumps and falls, or surgical attempts to remove heterotopic bone.[7,12–14] In the absence of trauma in FOP, HO forms a characteristic anatomic and temporal progression.[15] This ectopic bone formation in FOP, which generally is

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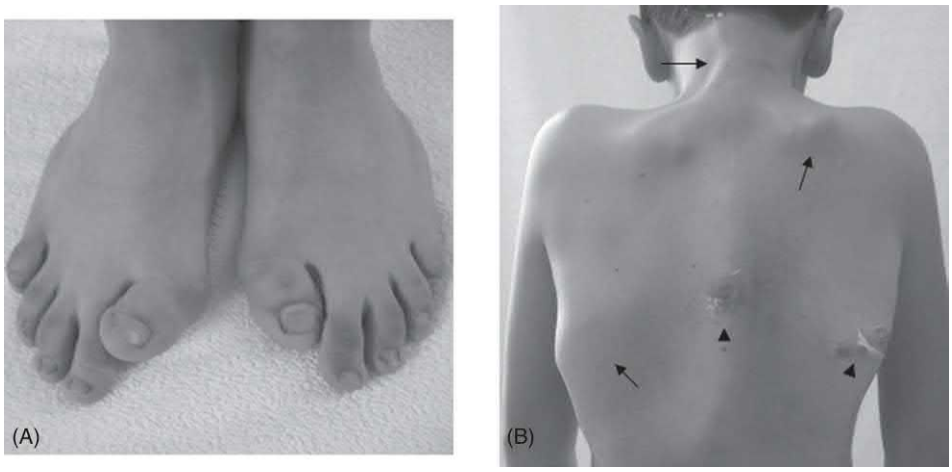

FIGURE 30.1 Diagnostic features of fibrodysplasia ossificans progressiva (FOP). (A) Photograph of the feet of a child with FOP showing characteristic short great toes and associated hallux valgus. (B) Photograph of the back of a child with FOP showing heterotopic ossification on the back and neck *(arrows)*. Although heterotopic ossification forms in deeper connective tissues and not in the skin, skin breakdown due to points of contact and pressure can occur *(arrowheads)*. Bone formation is preceded by tumor-like swellings in these locations.

asymmetrically distributed, is usually seen first in dorsal, axial, cranial, and proximal regions of the body with early HO most commonly in the neck, spine, and shoulders, and then later in ventral, appendicular, caudal, and distal regions. By the 3rd decade of life, most body regions are affected.[10,16]

The heterotopic bone formation in FOP is episodic and months or years of disease quiescence is common, although episodes can also occur in rapid succession.[2] Although the age of onset and rate of bone formation are highly variable among patients, complete ankylosis and permanent immobility will eventually occur as the ectopic bone becomes more extensive within muscles and connective tissues and bridges across joints.[16–18] Heterotopic bone in FOP exhibits normal modeling and remodeling based on radiographs, histopathology, and bone scans.[6,19,20]

2.2 Histopathology of FOP Lesions

Since tissue trauma has the risk of stimulating episodes of FOP lesion formation, biopsies are not obtained following diagnosis. However, early stages of FOP have been frequently misdiagnosed and biopsied. These tissue samples revealed that the histological stages of FOP lesions involve an initial catabolic phase of tissue destruction and turnover followed by an intense anabolic phase involving tissue formation and replacement.[6–8,21,22]

In early stages, FOP lesions are intensely hypoxic and show the presence of monocytes, macrophages, mast cells, and B- and T-lymphocytes that accompany destruction of the connective tissue integrity.[7,23] The presence of these cells, together with turnover of skeletal muscle, flare-ups following viral infections, the intermittent timing of flare-ups, and the suppression of early flare-ups in response to corticosteroids, support involvement of the immune system in the pathogenesis of FOP lesions.[12,24–26]

Following the catabolic phase, the anabolic phase begins with intense fibroproliferation that is associated with robust angiogenesis and neovascularity.[7,21] The lesion then matures through a typical process of endochondral ossification in which the fibroproliferative tissue transitions into an avascular cartilage stage followed by revascularization and osteogenesis. The resulting heterotopic bone is histologically mature lamellar bone tissue that may contain marrow elements.

2.3 Skeletal Development in FOP

In addition to HO, patients with FOP show discrete skeletal malformations, indicating that the underlying gene mutation influences formation of the skeleton during embryonic development in addition to directing cell fate decisions toward chondrogenesis and osteogenesis in adult soft connective tissues.[3]

Malformation of the great toes characterized by fibular deviation, also called hallux valgus, is the most consistent skeletal anomaly and this feature has been used to diagnose FOP prior to the onset of heterotopic bone formation.[25] These bilateral malformations are the results of abnormal formation of first metatarsals, proximal phalanges, and interphalangeal joints. The proximal phalanx of the first toe is aberrantly shaped particularly in the tibial aspect,[27] typically broader than normal, and often fused with the distal phalanx.[28] Additionally, these phalanges feature an extraosseous body that often fuses to the first metatarsal, likely contributing to the condition of hallux valgus.[27] Some children have an intact interphalangeal joint in the great toe at birth that fuses early in life with loss of articular

TABLE 30.1 FOP Phenotypes: Variable Expressivities

Diagnostic criteria for classic FOP:
Characteristic malformations of great toe (monophalangism, hallux valgus, and/or malformed first metatarsal);
progressive HO.
Common variable FOP features:
Found in 50%–90% of all FOP patients;
conductive hearing impairment;
cervical spine malformations;
proximal medial tibial osteochondromas;
short broad femoral necks;
thumb malformations (short first metacarpal, +/− monophalangism).
Additional occasional features of FOP variants:
Severe variable reduction deficits of digits;
absent finger/toe nails in digits with severe reduction deficits;
normal or minimal changes in great toes;
intraarticular synovial osteochondromatosis of hips and DJD of hips;
sparse, thin scalp hair (more prominent in 2nd decade);
mild cognitive impairment.
Occasional FOP-plus features:
Most identified in single patients;
severe growth retardation;
cataracts;
retinal detachment;
childhood glaucoma;
craniopharygioma;
persistence of primary teeth in adulthood;
anatomic abnormalities of cerebellum;
diffuse cerebral dysfunction with seizures;
polyostotic fibrous dysplasia;
primary amenorrhea;
aplastic anemia;
hypospadias;
cerebral cavernous malformations;
gonadal dysgenesis.

cartilage and intraarticular ankylosis. This highly reproducible clinical sign has been used to successfully diagnose FOP, even in utero.[29]

Approximately half of patients with a classic clinical presentation of FOP have malformed thumbs, although the degree of malformation in thumbs is less severe than in the great toe.[28] One study suggested that up to 85% of patients have short thumbs bilaterally. Although ankylosis in the thumbs was not reported for patients in this study,[30] it has been noted in others.[3] Malformations of other skeletal elements are more variably observed (Table 30.1).[3,31] The most common of these developmental abnormalities are short, broad femoral necks, and narrow cervical vertebra in the lateral dimension.

Malformations of the cervical spine are seen in nearly all classically-affected FOP patients and include large posterior elements, tall, narrow vertebral bodies, and variable fusions of the facet joints between C2 and C7.[31] Children with FOP often have neck stiffness and decreased range of motion prior to heterotopic bone at that site and have been misdiagnosed as Klippel–Feil syndrome (MIM #118100; http://omim.org/entry/118100), a disorder caused by inactivating mutations in the *GDF6* gene, a member of the transforming growth factor β (TGFβ)/bone morphogenetic protein (BMP) family of proteins.

Many with FOP develop early degenerative joint disease at both weight-bearing and nonweight-bearing joints, such as elbows, wrists, fingers, hips, knees, and feet. Early degenerative changes also occur in temporomandibular joints (TMJs), cervical facet joints, and costovertebral joints. Interestingly, FOP patients with less severe HO often develop severe degenerative joint changes early in life.[16]

Osteochondromas (benign osteochondral neoplasms or orthotopic lesions of skeletal remodeling) are a common feature of FOP.[32] Proximal medial tibial osteochondromas are most frequently observed, however, osteochondromas are often detected at other skeletal sites. The osteochondromas are usually asymptomatic, frequently bilateral, and most often pedunculated.[33] Osteochondromas occur in only a few hereditary disorders, the most common being multiple hereditary exostoses (EXT, previously MHE; MIM#133700), an autosomal dominant condition characterized clinically by numerous osteochondromas. MHE is caused by mutations in

the *EXT1* or *EXT2* genes,[34] which encode glycosylstransferases involved in the biosynthesis of heparan sulfate, an essential component of heparan sulfate proteoglycans (HSPG). HSPGs bind many cell surface receptors (including BMP receptors) and modulate signaling and multiple cellular processes.[35] HSPG modulation of BMP signaling is altered in cells from FOP patients, suggesting a potential common mechanism for these similar phenotypes.[35,36]

2.4 Additional Clinical Features of FOP

Some skeletal muscles, including the diaphragm, tongue, and extraocular muscles, are spared from FOP. Cardiac muscle and smooth muscle also are not affected by HO.[16] Ankylosis of the temporomandibular joint is common in later stages of FOP disease progression, as is submandibular HO.[37]

Hearing impairment occurs in about half of the FOP patients. Onset is usually in childhood or adolescence with slow progression. Hearing loss is most often conductive, possibly caused by middle ear ossification; however, the hearing impairment is neurologic in some patients.[38]

Patients with FOP frequently develop thoracic insufficiency syndrome (TIS), a life-threatening complication of cardiopulmonary function that can cause pneumonia and right-sided heart failure. Considering the role of *ACVR1* in cardiac development, TIS may be the result of chest wall constriction exacerbating minor and preexisting cardiac defects; however, it is yet to be investigated.[39,40] In FOP, TIS is associated with costovertebral malformations, ankylosis of the costovertebral joints, HO, and progressive spinal deformity.[41–44] TIS is the most common cause of death in FOP, with the median age of death for patients being 40 years.[44]

Fractures of normotopic skeletal bone are not increased in patients with FOP; and when fractures occur, they are not associated with the formation of new HO. However, healing of fractured heterotopic bone appears to be accelerated.[45]

Routine biochemical assessment of bone and mineral metabolism is usually normal in FOP patients, although increased serum alkaline phosphatase activity and erythrocyte sedimentation rate have been observed during disease activity. Elevated urinary basic fibroblast growth factor levels have also been correlated with disease flare-ups during the preosseous angiogenic phase of fibroproliferative lesions.[46]

2.5 Atypical FOP Phenotypes

Among patients with FOP-type progressive HO, occasional cases are associated with clinical features unusual for FOP[3] (also see Section 3.2 describing ACVR1 mutations in atypical FOP). Two groups of these atypical FOP patients have been described. "FOP-plus" patients have the classic defining FOP features of progressive endochondral HO and great toe malformations with one or more features that are uncommon in FOP patients (Table 30.1). These uncharacteristic phenotypes may have variable effects directly caused by the underlying mutation; however, a direct link between the *ACVR1* mutations and the phenotypes has not been established. Thus, the possibility that they are either secondary to primary effects, such as HO or independent of FOP remains. "FOP variants" clinically present with significant deviations from one or both of the two classic defining features of FOP, most notably more or less severe malformations of the digits. Table 30.1 lists the clinical features that have been recognized in classic, FOP-plus, and FOP variant patients.

3 GENETICS AND MOLECULAR GENETICS OF FOP

The severe physical disability of FOP results in low reproductive fitness. As a result, genetic transmission rarely occurs and most cases of FOP are caused by de novo mutations.[47,48] The population frequency of FOP is estimated to be about 1 in 1.5–2 million.[16,47] No gender, racial, ethnic, or geographic differences or clustering are observed.[47]

Nine families with inheritance of FOP (eight families with classic FOP and one FOP variant family) have been reported,[3,47,48] and show an autosomal dominant inheritance pattern indicating that one mutant allele is sufficient to cause the disease (Fig. 30.2). Inheritance can be maternal or paternal, and male-to-male transmission has been observed.

Three pairs of monozygotic twins with FOP revealed that congenital toe malformations differed among twin pairs, but were identical within each pair, supporting that additional genetic components influence the effects of the *ACVR1* gene mutation on skeletal development.[49] However, each member of the FOP twin pairs had divergent patterns and timing for episodes of postnatal HO that varied with life history, including tissue trauma and viral infections. This twin study[49] supports the *ACVR1* gene mutation in concert with genetic modifier genes are a major influence on the disease phenotype during prenatal development; while environmental factors or postnatal epigenetic changes are the key determinants of the postnatal progression of HO.

3.1 ACVR1 (ALK2) R206H Mutations in FOP

In 2006, whole genome genetic linkage analysis and positional cloning using five families with a classic

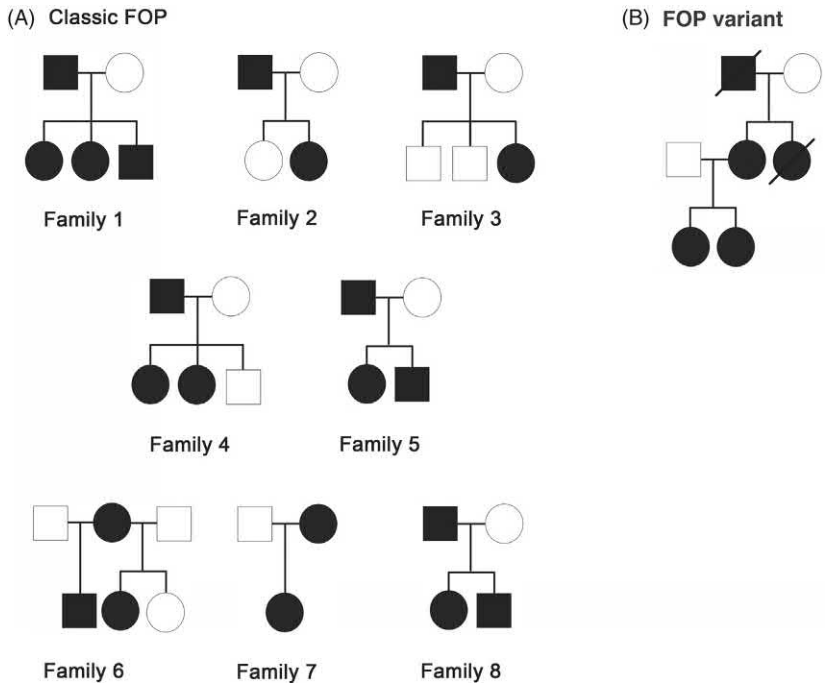

FIGURE 30.2 Pedigrees showing inheritance of FOP. (A) Classic FOP. The genetic inheritance from eight individuals with the classic ACVR1^{R206H} mutation has been identified and the family pedigrees are shown. Families 1–5 were used for genetic linkage studies[48]. (B) FOP variant. Inheritance of an FOP variant mutation (G328R ACVR1) was identified in the family indicated. Genetic transmission through three generations of the family is suspected based on clinical exam; however, the grandfather and maternal aunt (symbols with a diagonal line) were deceased at the time of DNA sequence analysis and could not be confirmed. *ACVR1* mutation analysis of the family was reported[3].

clinical presentation of FOP identified *ACVR1* (Activin A type I receptor) as the mutated gene in FOP.[48] The *ACVR1* gene encodes a type I receptor for BMPs that is also known as ALK2 (Activin-like kinase 2) (throughout the text, *ACVR1* will be used for the gene and ALK2 to identify its protein product). All sporadic and familial cases with classic clinical features of FOP (progressive HO that initiates during childhood or adolescence and characteristic great toe malformation) (Fig. 30.1) harbor a heterozygous single nucleotide substitution (c.617G > A) that changes amino acid 206 from arginine to histidine (R206H).[3,48] Codon 206 occurs within the glycine-serine (GS) region of the cytoplasmic domain of ALK2 (Fig. 30.3) and is highly conserved among species. Although there is a high degree of amino acid sequence conservation among the three human BMP type I receptors (ALK2, ALK3, and ALK6), ALK2 codon 206 normally encodes arginine while the corresponding amino acid in ALK3 and ALK6 is a lysine (Fig. 30.4). Arginine, lysine, and histidine are all positively charged amino acids; however, histidine is structurally distinct from the other basic residues and substitution with this amino acid is likely to alter tertiary protein structure and molecular interactions at codon 206 in the receptor.[50,51]

The *ACVR1^{R206H}* mutation is fully penetrant; that is, no "silent carriers" of the mutation have been identified. Current data support that there is no locus heterogeneity in FOP.[3] In 2001, mutations in the BMP antagonist Noggin were reported as the genetic cause of FOP,[52] but could not be verified in 18 sporadic and 4 inherited FOP cases, which were subsequently confirmed to have *ACVR1* mutations.[53,54]

3.2 *ACVR1* Mutations in Atypical FOP

Patients who have FOP-type progressive HO in combination with atypical clinical features for FOP (Table 30.1) have been examined for *ACVR1* mutations and all have heterozygous *ACVR1* missense mutations in conserved amino acids.[3,4,55–61] The c.617G > A; R206H mutation that occurs in all cases of classic FOP has also been found in most cases of FOP-plus.[3] However, non-R206H *ACVR1* mutations were identified in each of the FOP variants and in four cases of FOP-plus (Table 30.2).

All *ACVR1* mutations identified in classic FOP, FOP-plus, and variant FOP are single-nucleotide substitutions causing missense mutations, with one exception of a 3-nucleotide deletion spanning two codons that results in replacement of two amino acids with a single amino acid[3] (Table 30.2). No nonsense or frameshift mutations have been found, supporting that FOP mutant ALK2 proteins are synthesized. The identified mutations occur

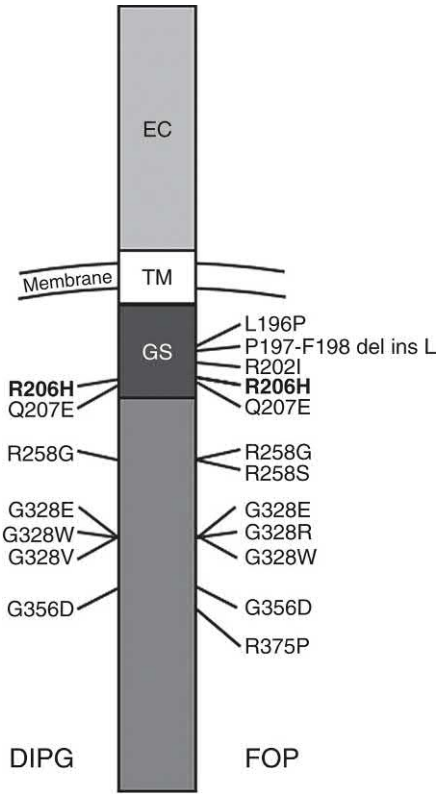

FIGURE 30.3 ALK2 mutations in FOP and diffuse intrinsic pontine gliomas (DIPG). ALK2, the transmembrane type I BMP receptor protein encoded by the *ACVR1* gene, is comprised of four domains: an extracellular ligand binding domain (EC), a transmembrane domain (TM), a glycine-serine (GS) rich activation domain, and a protein kinase domain (PK). Twelve mutations (listed at right) have been identified in ALK2 in FOP, all resulting in a functional protein. Mutations occur within the GS activation domain or the PK domain, which are both located intracellularly but not associated with ligand binding. The classic FOP mutation (R206H), which occurs in greater than 95% of patients with FOP, resides within the GS domain, a domain that is predicted to function intracellularly as an "on/off" switch. Variant FOP mutations have been identified within both the GS and the PK domain, and are predicted to affect binding affinity to SMAD effector proteins and receptor auto-phosphorylation. Seven mutations (listed at left) have been identified in ALK2 in DIPG tumors, all of which have previously been identified in FOP patients, except G328V. These mutations would result in the same enhancement of BMP pathway signaling, as seen in non-neuronal cell lineages expressing ALK2 mutations. Patients with DIPG also present with somatic heterozygous mutations in other proteins, such as Histone H3; ALK2 mutations alone do not induce a tumorigenic phenotype.

in either the glycine-serine (GS) activation domain or the protein kinase domain (Fig. 30.3), which are regions of the ALK2 receptor that are important in downstream signal transduction.[3,51,62] Each of the mutated amino acids in the FOP variants and FOP-plus patients is evolutionarily conserved and also invariant among all three human type I BMP receptors.[3,4,55–60] Protein structure homology modeling predicts that these amino acid substitutions activate the ALK2 protein to enhance receptor signaling.[3,56] None of the identified *ACVR1* mutations have been found in unaffected individuals (with the exception of rare somatic mutations in some tumors; see Section 3.4), and all patients with FOP-type heterotopic endochondral ossification so far examined have a mutation in the *ACVR1* gene.[3] *ACVR1* DNA sequencing has been used to confirm the diagnosis of FOP that was suspected based on the appearance of great toe malformation prior to initiation of HO.[25]

3.3 Phenotypic Variability

Progressive heterotopic endochondral ossification is the common feature shared by all classic FOP, FOP-plus, and FOP variant patients, supporting that all of the identified *ACVR1* missense mutations direct postnatal induction of cartilage and bone cell differentiation. However, the age of onset, rate of progression, and severity of HO vary greatly. A late onset of HO is often, but not consistently, associated with a slow or mild progression of the subsequent ectopic bone formation.[3] Among the identified *ACVR1* mutations, there is limited genotype–phenotype correlation.[3,4,55–60]

FOP variant patients show a significant range in severity of their great toe malformations (from normal toes to a nearly complete absence of digits), and genotype–phenotype correlations may explain at least some of these differences.[3] The most severe malformations are associated with specific amino acid substitutions in codons 258, 328, and 356 (Table 30.2). As the FOP variant *ACVR1* mutations occur at different positions within the resulting mutant proteins, the specific molecular mechanisms or strength of downstream signaling effects may differ.[51,63] However, given the rarity of individual variant *ACVR1* mutations, most of which have been found in only one or two FOP-plus and/or FOP variant patients, such cause and effect correlations are currently difficult to establish.

The extremes of the range of phenotypic variability in patients with heterotopic endochondral ossification are seen in FOP variant patients.[3,4,55–60] However, variable expressivity of phenotypes among classic FOP patients with the $ACVR1^{R206H}$ mutation is also observed.[3,48] Features noted in some but not all cases of classic FOP include hearing loss, osteochondromas, and thumb and other digit malformations (described earlier and Table 30.1). Variability in the degree of great toe malformations (although within a narrower range than observed for FOP variants) and the onset and rate of progression of HO are also observed in classic FOP patients.

3.4 *ACVR1* Mutations in Diffuse Intrinsic Pontine Gliomas (DIPGs)

ACVR1 mutations have been identified outside of the context of FOP in patients with diffuse intrinsic pontine

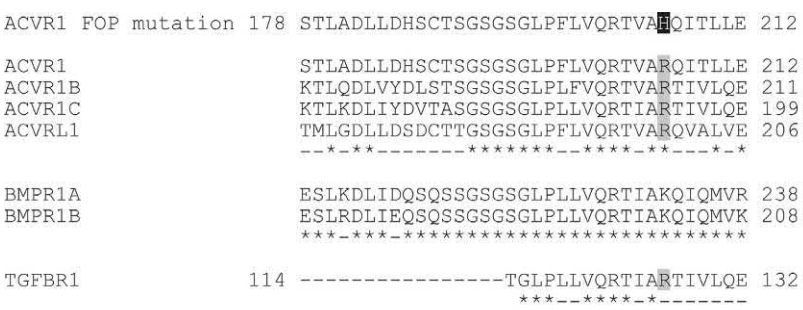

FIGURE 30.4 Type I receptors in the transforming growth factor β (TGFβ)/bone morphogenetic protein (BMP) family show amino acid sequence conservation in the GS domain. At the position analogous to ACVR1 codon 206, there is an arginine (R) in the type I Activin receptors (ACVR1, ACVR1B, ACVR1C, ACVRL1) and TGFβR1. By contrast, two other BMP type I receptors (ALK3/BMPRIA and ALK6/BMPRIB) have a lysine (K) at this position. Like arginine, lysine is a positively charged amino acid and is expected to maintain similar function; however this amino acid difference may contribute to receptor specificity and differences in regulation of downstream signaling. *GenBank Accession numbers*: Human proteins for ACVR1B (Q61271); ACVR1C (NP_660302); ACVRL1 (NP_000011); BMPRIA (NP_004320); BMPRIB (AAH47773); and TGFBR1 (AAH71181). *Source: Reproduced from Shore et al., 2006; Suppl. Fig. 2.*

TABLE 30.2 ACVR1 Variant Mutations in FOP

ACVR1/ALK2 mutations				Heterotopic ossifcation[a]		
Nucleotide	**Amino acid**	**#Cases**	**Toe malformations**	**Onset**	**Progression**	**References**
c.587T > C	L196P	1	None	Late	Mild/slow	4
c.590-592 del CTT	P197-F198 del ins L[b]	1	None	Late	Rapid	3
c.605G > T	R202I	2	Minor to none	Late	Mild-characteristic	57,60
c.619C > G	Q207E	1[c]	Characteristic	Early	Characteristic	3
c.772G > A	R258G	2	Severe	Early	[d]	61
c.774G > C	R258S	2	Minor to none	Characteristic	Mild-characteristic	56
c.774G > T	R258S	1	Characteristic	Characteristic	Characteristic	59
c.982 G > A	G328R	2[e]	Minor to none	Late	Variable	3
c.982 G > C	G328R	1	Minor	Late	Mild	3
c.982 G > T	G328W	2	Severe	Characteristic	Characteristic	3
c.983 G > A	G328E	3[f]	Severe	Characteristic	Characteristic	3,57,58
c.1067 G > A	G356D	5[c]	Severe to Characteristic	Early-late	Characteristic	3,55
c.1124 G > C	R375P	1	None	Late	Mild/slow	3

[a]*The typical age range of classic FOP is 2–8 years; early onset is defined as before 2 years of age and late onset after 10 years. Characteristic severity and rate of progression is relative to patients with classic FOP and the R206H mutation.*
[b]*A 3-nucleotide deletion across codons 197 and 198 replaces 2 amino acids with a single in-frame leucine (L).*
[c]*The patient with a Q207E mutation and one patient with a G356D mutation have been described as FOP-plus (Kaplan et al., 2009). All other patients are described as FOP variants.*
[d]*Both patients with R258G mutation are too young to determine progression.*
[e]*One sporadic case and one family (with three affected members) have been identified.*
[f]*The patient described in Petrie et al.[57] is the same as one of the two patients reported in Kaplan et al. (2009).*

gliomas (DIPGs).[64,65] DIPGs are a rare class of brainstem gliomas and the leading cause of death among all pediatric brain tumor patients.[66] New methods of tumor biopsy and rapid retrieval of autopsy material have increased the availability of DNA, RNA, and primary cell lines from these gliomas, allowing for major advances in our understanding of DIPG biology. DIPGs are associated with gross chromosomal abnormalities and somatic heterozygous mutations in histone H3 isoforms.[67–69] Four recent studies examining over 200 DIPG patients also identified recurrent heterozygous somatic missense mutations in *ACVR1* in about 33% of DIPGs. These mutations

were specific to brainstem gliomas.[67–70] Seven mutations were identified: R206H, Q207E, R258G, G328E, G328V, G328W, and G356D (Fig. 24.3), and except for G328V all have also been identified in FOP patients.[67–70] As with FOP, each *ACVR1* mutation in DIPG was shown to increase basal canonical BMP pathway signaling in vitro in mouse primary astrocytes. However, the *ACVR1* mutation alone does not induce a tumorigenic phenotype. Work is underway to determine how the mutations in *ACVR1* contribute to DIPG pathogenesis and how this finding can be implemented in treatment strategies.

4 ANIMAL MODELS

An ideal in vivo model for FOP would recapitulate the complete FOP phenotype, including the characteristic skeletal malformations and progressive heterotopic endochondral bone formation. Understanding the specific developmental effects of the $ACVR1^{R206H}$ mutation will require construction and analysis of genetically modified animal models. Knock-in mouse models for FOP have been developed;[71–73] however, various animal models in which HO can be induced, through genetic or nongenetic means (described further), also provide important information for understanding the pathophysiology of FOP, HO, and endochondral bone formation, as well as providing key information about the activity and effects of the BMP signaling pathway in vivo.

4.1 In Vivo Models for BMP Signaling

The evolutionarily conserved BMP signaling pathway is studied in several highly informative animal models, including fruit flies (*Drosophila melanogaster*) and zebrafish (*Danio rerio*), providing important insight into the cellular and molecular mechanisms of BMP signaling and the activities of the ALK2 receptor or its orthologs in vivo.[74–76] BMP signaling in mammalian systems has been examined predominantly in the mouse to investigate its molecular mechanisms and developmental consequences.[77]

4.1.1 Drosophila and Mutations in Sax

The BMP pathway is highly evolutionarily-conserved,[78] and although flies do not form bone, *D. melanogaster* (fruit fly) is an informative genetic system for understanding the components and mechanisms of the BMP signaling pathway.[79,80] Relevant to FOP, mutations in *saxophone* (*sax*), a *Drosophila* BMP type I receptor gene that is the ortholog of the human ALK1/ACVRL1 and ALK2/ACVR1 receptors, have been identified and examined for effects on BMP signaling and in vivo function.[81] Although a *sax* mutation in *Drosophila* corresponding to human $ACVR1^{R206H}$ has not yet been reported, a *sax* G412E mutation (sax^2) occurring in the amino acid corresponding to ALK2 codon 356, which is mutated in some FOP variants, participates in BMP receptor complexes and produces elevated signaling.[81]

4.1.2 Zebrafish Models and the Alk8 Receptor

Investigations using zebrafish embryos have demonstrated that a BMP signaling gradient is critical for patterning cell fates to establish dorsal–ventral polarity, with high levels of BMP signaling inducing ventral tissues/structures and low levels inducing dorsal structures in vertebrates.[82] Zebrafish embryos develop dose-dependent phenotypes in response to changes in BMP signaling.[83–85] Developing zebrafish embryos therefore provide a sensitive assay for perturbations in the BMP signaling gradient by evaluating dorsal/ventral patterning.

Zebrafish Alk8, the paralog of human ALK2, is a BMP type I receptor required for signaling during dorsal–ventral axis formation.[83] *Alk8* null embryos become dorsalized (form head structures and lack tail structures), but can be rescued to a normal phenotype by injection of wild type human ALK2 mRNA. This indicates that the wild type receptor restores normal BMP signaling levels.[86] By contrast, injection of mutant $ACVR1^{R206H}$ mRNA into *alk8* null embryos induced a "hyperventralized" phenotype (absence of head structures), demonstrating that the mutant $ACVR1^{R206H}$ present in FOP results in increased BMP signaling relative to wild type ALK2 in a much broader context than HO alone.[86]

An additional advantage provided by the zebrafish system is the use of embryonic development assays in high throughput screening.[87,88] The chemical compound dorsomorphin, a small molecule inhibitor of all BMP type I receptors (ALK2, ALK3, and ALK6), was identified by in vivo screening for effects on zebrafish embryonic dorsal–ventral polarity.[85] More specific small molecule inhibitors that selectively target single BMP type I receptors, including ALK2, have been developed in recent years.[89,90] Furthermore, transgenic zebrafish models also have great potential as a system to investigate bone and skeletal development and formation of heterotopic bone.[91,92]

4.1.3 BMP Signaling in Genetically-Engineered Mouse Models

Genetically-modified mice have been highly informative in increasing our understanding of the cells and tissues that express BMP ligands and receptors, as well as identifying the consequences of increased or decreased BMP signaling in specific tissues and organ systems.[78] Transgenic and knockout models have demonstrated that BMP signaling is active during skeletal development and repair, as well as in a wide range of nonskeletal tissues.[78] However, despite the effects of BMP implant experiments showing that increased BMP signaling can

induce ectopic bone formation in extraskeletal tissues, most models of overexpression of BMP ligands or receptors that would be expected to increase BMP signaling do not cause HO.[92–94]

The BMP pathway is regulated, in part, through extracellular and intracellular antagonists, and genetic deletion of BMP inhibitor proteins indirectly increases BMP signaling.[95] Noggin is an extracellular antagonist that acts by binding BMP ligands and preventing the interaction of BMPs with the extracellular domain of the receptors.[50,96] Homozygous knockout of *Noggin* in mice is perinatal lethal due to severe congenital skeletal defects.[97,98] Skeletal analysis of Noggin null mice revealed congenital HO, multiple synostoses, ankylosis of the chest wall, lack of joint formation,[97,98] and spine malformations that are similar to those in FOP patients.[31] While targeted disruption of other extracellular BMP inhibitors, such as Chordin, Dan, and Gremlin, cause abnormal skeletal phenotypes,[92–94] none of these cause an FOP-like phenotype or HO.

Smad6 and Smad7 are intracellular inhibitory proteins of TGF-β/BMP signaling.[99–101] In humans, decreased expression of Smad6 results in increased BMP signaling and calcification of the fibrosa endothelium of the aortic valves.[102,103] Targeted gene disruption and tagged expression of murine Smad6 demonstrated expression of Smad6 in the heart, blood vessels, and participation in aortic ossification, although no other ectopic ossification was found.[104] Targeted disruption of Smad7 in mice caused defects in cardiovascular development, ventricular septal defects, outflow tract malformations, and impaired cardiac functions and arrhythmia.[105] Mice deficient for the BMP receptor antagonist FKBP12 do not form heterotopic bone, and, like disruption of Smad6 and Smad7, exhibit cardiac defects.[106] That the main phenotypic effects of loss of negative regulators of BMP receptor signaling, such as Smads 6 and 7 and FKBP12, are cardiac phenotypes, which raises speculation that the impaired cardiac function associated with TIS in FOP could be a primary effect of *ACVR1* mutation and not only secondary to HO and skeletal fusions.[43] These genetic models also indicate the sensitivity of cells participating in heart development and function to BMP signaling.

4.2 BMP Induction of Endochondral Ossification in Nongenetic and Genetic Models

Pioneering studies[107] led to the identification of BMPs as a family of proteins with the unique capacity to induce the entire program of endochondral bone formation in vivo. Although the genetically modified mouse models described earlier do not induce a robust heterotopic bone forming phenotype, other models have been successful in inducing ectopic bone formation.[92] Mouse models that have been used in recent investigations are described later.

4.2.1 Nongenetic Induction of HO: BMP Implants

Directly introducing recombinant BMP proteins to in vivo sites was an initial approach used to develop models of HO. These studies demonstrated that a threshold level of BMP protein is necessary to induce HO, and that a carrier substance (such as deactivated bone matrix, collagen sponges, Matrigel, scaffolds, or nanoparticles) is required to prevent diffusion of BMP and maintain a sufficient local concentration to induce bone formation.[21,92,108] The resulting heterotopic endochondral bone formation replicates the histological stages that are observed in human FOP and the formation of mineralized bone.[21] Local increases of BMP protein and HO have also been obtained though injection of cells expressing plasmid or viral constructs that overexpress BMPs.[109,110]

4.2.2 Transgenic Mice Overexpressing BMP: Nse-BMP4

As noted earlier, transgenic overexpression of BMPs driven through a variety of different promoters has not been associated with induction of heterotopic bone formation.[92] An exception is a transgenic mouse that specifically overexpresses BMP4 under control of the neuron-specific enolase (Nse) promoter.[111] Nse-BMP4 mice show FOP-like HO with bone formation through an endochondral process.[111] Double transgenic mice overexpressing both Noggin and BMP4 through the *Nse* promoter showed full rescue of heterotopic bone formation.[111]

4.2.3 Constitutively Active Alk2 Mice ($Alk2^{Q207D}$)

Mice that are deficient for both Alk3 and Alk6 in chondrocytes show severe chondrodysplasia and impaired endochondral ossification.[112] Chondrocyte-specific overexpression of a ligand-independent constitutively active *Alk3* (*Bmpr1a*) allele using the *Col2* promoter induced severe skeletal malformation (including fusion of joints, particularly of the tibia, femur, and patella) and confirmed a role for BMP signaling through this receptor in skeletal development; however, overexpression of the Alk3 receptor did not induce HO.[113] In this model, it is possible that the *Col2* promoter activates expression of the constitutively active receptor at too late a stage of chondrogenesis, and that heterotopic osteogenesis could be induced through Alk3 activation earlier in the differentiation process.

Global constitutive activation of Alk2 induces embryonic lethality;[114] however, transgenic mice containing a Cre recombinase-inducible constitutively active allele, $Alk2^{Q207D}$, have been developed.[114] $Alk2^{Q207D}$ confers ligand-independent constitutive activity of the Alk2 receptor and increased BMP signaling. While global Cre-induced postnatal constitutively active Alk2 expression did not yield HO,[115] intramuscular injection of Cre-expressing adenovirus and cardiotoxin to

stimulate muscle injury induced ectopic endochondral bone formation, ankylosis, and functional impairment at the site of injection.[115,116] Recent experiments expressing *Alk2*^{Q207D} in *Nfatc1*-positive cells showed that the mutation induces ectopic bone formation primarily in the distal joints.[117]

4.2.4 *Acvr1 R206H Knock-In and Conditional Knock-In Mice*

The development and characterization of an *Acvr1* R206H (c.617G > A) knock-in mouse (*Acvr1*^{R206H}) provided the first direct in vivo evidence that the R206H mutation in ACVR1 causes FOP.[73] Although germline transmission of this mutation is perinatal lethal, phenotypic characterization of mice that are chimeric for *Acvr1*^{R206H} cells showed recapitulation of every clinical feature of patients with classic FOP, including embryonic skeletal malformations and postnatal heterotopic endochondral bone formation.[73] In addition, histological analyses of regions undergoing HO demonstrated the same progression of cellular events as observed in patient lesions, including inflammation-associated catabolism of connective tissues followed by a robust anabolic tissue replacement by cartilage and bone.

Recently, a conditional knock-in mouse (*Acvr1*$^{cR206H/+}$) with expression of *Acvr1*^{R206H} controlled by Cre-inducible recombination has been reported.[71,72] Global expression of the conditional *Acvr1*^{R206H} mouse allele postnatally avoids perinatal lethality by germline induction and allows for cell-specific expression of the R206H mutation. Mice induced to globally express *Acvr1*^{R206H} postnatally reproducibly form HO in response to muscle injury.[71] Mice expressing the R206H mutation only in limb mesenchymal progenitor cells (*Prrx1*$^{+}$) formed skeletal malformations similar to those seen in patients, with altered chondrocyte development in growth plates, and postnatal HO in the absence of injury.[71]

4.3 Nongenetic Injury-Induced Models of Heterotopic Ossification

An inflammatory component is a common feature among most nongenetic incidents of HO.[26] Development of HO following joint arthroplasty,[118,119] combat blast injury,[120,121] burn,[122] and tendon/ligament injury and reconstruction[123–127] occurs with moderate frequency, with estimates as high as 65% for select pathologies.[122] Significant efforts have recently focused on developing animal models replicating these nongenetic, injury-induced episodes of HO to better understand disease etiology and assist with candidate drug testing. A combination burn/tenotomy HO murine model was recently developed[128] and has been used to identify SMAD7, a BMP signaling antagonist protein, as a factor that prevents HO formation in Achilles tendon injury.[129] Additionally, a recently developed blast injury rat model of HO[130] will be important for investigating severe trauma-induced HO development.

4.4 Naturally Occurring Animal Models of Heterotopic Ossification

To our knowledge, no naturally occurring mice with HO have been identified. However, there have been a few reports of spontaneous naturally developing FOP-like conditions in other animals.[92,131–138]

Several reports have documented ocular HO in guinea pigs.[139–141] The HO appears as a white lesion at the limbus, which corresponds to new bone formation in the ciliary body of the eye and was initially reported as osseous choristoma.[139,141] The heterotopic bone formation includes formation of bone marrow and marrow elements within the ciliary bodies.[140]

Bone formation similar to FOP lesions in humans has been reported in two German shepherd dogs.[142] Radiography detected heterotopic bone in gluteal muscles and histopathological examination revealed proliferation of cellular fibrous tissue, osteoid tissue, and immature bone formation.[142] Sporadic incidences of dogs having cervical HO, thoracic limb lesions, and cranial distal scapular lesions morphologically similar to lesions in FOP have also been described.[133] Recently, a 6-year-old Doberman Pinscher was reported to have myositis ossificans in the semimembranosus muscle and histopathological examination revealed dense, fibrous connective tissue with bone formation. Central zones of the lesion showed mesenchymal-like cells, fibroblasts mixed with osteoblasts, and immature bone.[143]

The disease progression and lesion formation in case reports of cats with HO is highly similar to FOP.[7,136–138] Radiography and micro computerized tomography (μCT) imaging showed extensive HO.[136,138] Histology revealed intense perivascular lymphocytic infiltration, fibroproliferative lesion formation, and endochondral bone formation.[136,138]

Most cases of naturally occurring HO in animals have been reported prior to the identification of *ACVR1* mutations as the cause of FOP. Several cases of FOP-like HO have been reported in cats and are currently under investigation for *ACVR1* mutations.[138,144]

4.5 Osteochondromas and Mouse Models

Osteochondromas, benign osteochondral neoplasms or orthotopic lesions of skeletal remodeling, are detected in greater than 95% of classically-affected FOP patients.[32] Osteochondroma-like structures develop in a mouse model expressing *Acvr1*^{R206H}.[73] Multiple mouse models have been developed for osteochondromas through the heterozygous inactivation or mutation of the *Ext1* and

Ext2 genes.[32,145,146] Ext1 and Ext2 participate in heparan sulfate biosynthesis and regulate the hedgehog signaling pathway,[147,148] and have been implicated in chondrocyte maturation in the growth plate.[149] These mouse models of osteochondromas provide in vivo systems to investigate the signaling interactions between BMP and other pathways during osteochondroma formation, as well as the roles of interacting pathways in heterotopic endochondral bone formation.

5 FUNCTIONAL AND MOLECULAR PATHOLOGY

5.1 BMP Signaling and ACVR1/ALK2

BMP ligands signal through tetrameric complexes of two type I and two type II serine-threonine kinase receptors on the cell surface (Fig. 30.5).[93,94] When the ligand binds the extracellular region, type II receptors phosphorylate the glycine-serine (GS) domain of type I receptors. In turn, activated type I receptors activate downstream signaling through BMP pathway–specific SMAD1, SMAD5, and SMAD8 (SMAD1/5/8) proteins, as well as through MAP kinase pathways.[93,94] In addition to ALK2 (the BMP type I receptor mutated in FOP), BMP signal transduction is mediated through the BMPR1A/ALK3 and BMPRIB/ALK6 type I receptors, which have been implicated in skeletogenesis, as well as the ALK1 and ALK4 receptors.[93,94]

BMPs are members of the TGFβ family of signaling proteins.[150–152] The TGFβ/BMP family regulates a wide range of cellular activities, including differentiation, proliferation, apoptosis, migration, positional information, and stem cell renewal.[153–157] Distinct from other members of the family, many members of the BMP subgroup can induce the complete process of endochondral bone formation.[153] BMPs and their receptors are expressed throughout development and in many adult tissues, including skeletal muscle and cartilage.

5.1.1 Structural Modeling of Mutant ALK2 Receptors

Following identification of the R206H mutation in ALK2, structural homology modeling was used to provide initial information regarding the functional effects of the mutation on BMP signaling.[50] The amino acid sequences of the cytoplasmic domains of all TGFβ/BMP type I receptors are highly conserved, allowing for modeling of ALK2 based on the structure of ALK5. X-ray crystallography of human TβRI/ALK5 revealed the formation of an ion salt bridge between R203 (analogous to R206 in ALK2) and D266 (analogous to D269 in ALK2). In silico modeling of wild type ALK2 predicted formation of an identical salt bridge; however, this salt bridge that might allow for a pH-sensitive switch leads to ligand-independent constitutive activation.[50] The crystal structure of ALK2[51] indicates that FOP mutations alter interactions that stabilize the inactive state of the kinase domain, leading to inappropriate activation.

Structural modeling of the FOP variant mutations in ALK2 suggests that these amino acid substitutions (Fig. 30.3) also lead to receptor malfunction of the kinase domain.[3,4,51,56,57,94] Multiple ALK2 mutations, including the classic R206H mutation, disrupt key interactions with the BMP autoregulatory protein FKBP12 that stabilize the inactive site of the receptor.[51] Several substitutions of Glycine 328, which is within the kinase domain, have been identified in patients, each of which could possibly affect binding of Smad proteins or alter binding of FKBP12.[3,57] Other mutations identified in the protein kinase domain of ALK2 (G356D and R375P) may disrupt ion pair formation and promote phosphorylation of the receptor, leading to constitutive activity.[3]

5.1.2 BMP Pathway Signaling in FOP Patient Cells

The formation of HO following surgical resection of tissue is common in FOP patients; therefore following a diagnosis of FOP, surgical procedures are avoided, making it difficult to obtain samples for research purposes. Despite these limitations, minimally-invasive methods have been successfully used to obtain cells from FOP patients.[57,158,159] Blood samples can be safely drawn from FOP patients and used to establish lymphoblastoid cell lines (LCLs) from circulating peripheral–blood mononuclear cells.[158] These patient cell lines were used for a number of early studies on FOP prior to the discovery of the $ACVR1^{R206H}$ mutation and provided the first evidence of misregulated BMP pathway signaling.[57] Although LCLs were used to generate much useful information through BMP signaling assays, these cells are unable to provide insight into differentiation to bone or cartilage. However, more recently, stem cells from human exfoliated deciduous teeth (SHED cells)[160] of FOP patients have been used to examine the effects of $ACVR1^{R206H}$ on relevant biological processes, such as osteogenesis.[159]

In vitro experiments using LCLs and SHED cells from FOP patients revealed a consistent pattern of aberrant BMP signaling.[159,161,162] Although LCLs do not express detectable levels of BMP-SMAD-responsive proteins, FOP LCLs exhibit increased phosphorylated-p38 MAPK protein levels, a noncanonical BMP signaling pathway.[161,162] In addition, expression of *ID1* and *ID3*, both direct transcriptional targets of BMP signaling, was increased in FOP LCLs.[161,162] Similar experiments conducted using SHED cells isolated from FOP patients revealed dysregulation of both the canonical SMAD-dependent and the noncanonical p38 MAPK BMP signaling pathways.[159]

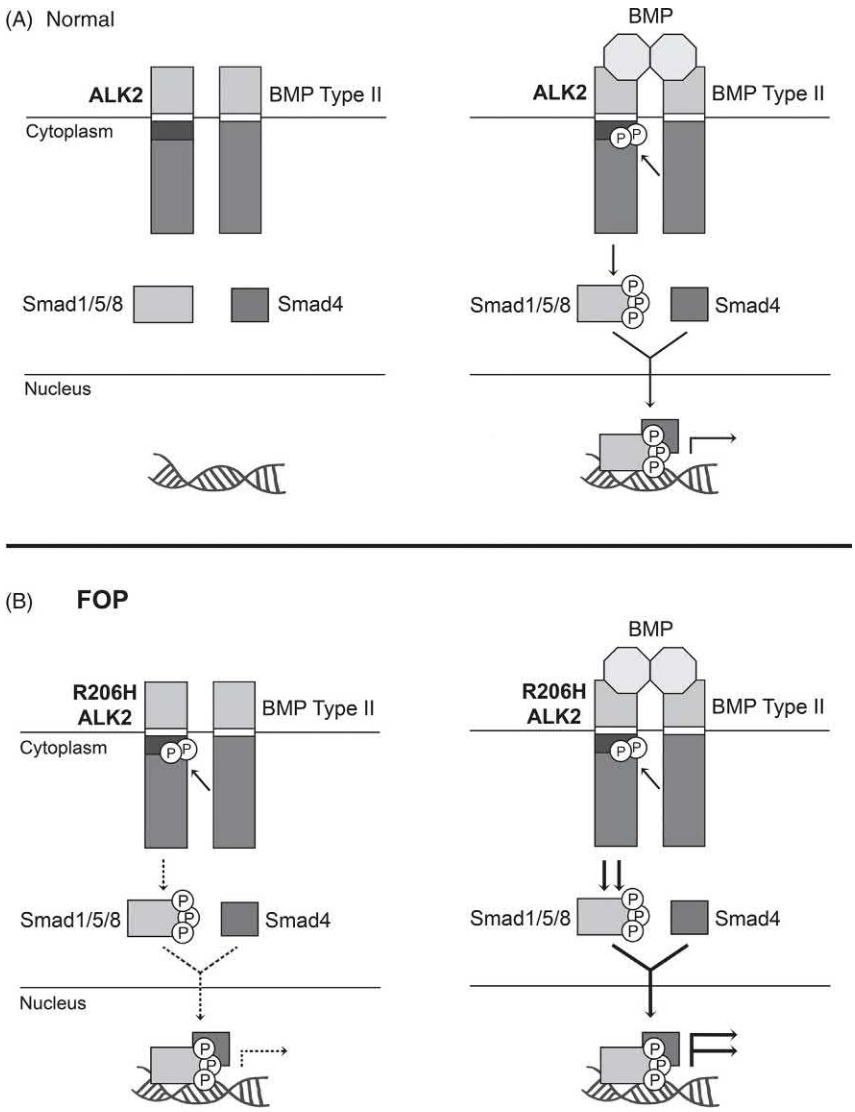

FIGURE 30.5 **BMP signaling through ALK2.** BMPs signal through heteromeric receptor complexes consisting of two type I receptors (ALK2 is shown here), two type II receptors, and ligand dimer. In this schematic, the type I dimer and the type II dimer are depicted by single bars. (A) In the absence of BMP ligand, signaling through normal ALK2 is silenced, SMAD1/5/8 proteins remain unphosphorylated and in the cytoplasm. Binding of BMP ligand causes a conformational change in ALK2, allowing the protein kinase domain of type II receptor to phosphorylate residues in the GS domain of ALK2. This activation of ALK2 enables phosphorylation of SMAD1/5/8 and nuclear translocation with co-SMAD4, followed by transcription of BMP responsive genes. Type I receptors, including ALK2, also activate noncanonical pathways, such as p38 MAPK (not shown here). (B) $ALK2^{R206H}$ exhibits leaky signaling and gene transcription in the absence of BMP ligand. $ALK2^{R206H}$ has been reported to require the type II receptor for activation. The composition of the relevant functional receptor complexes (type I receptor homodimers, heterodimers, or both) has not been established. In the presence of BMP ligand, signaling through $ALK2^{R206H}$ shows enhanced levels of phosphorylated SMAD1/5/8 and increased expression of BMP responsive genes relative to normal ALK2 signaling.

Elevated and prolonged cell-surface expression of the BMP type I receptor BMPRIA/ALK3 was observed in FOP LCLs as a consequence of reduced receptor degradation and internalization.[161] The mechanism through which mutations in one BMP type I receptor (ALK2) affects another (ALK3) is not yet understood.

5.1.3 Effects of ACVR1/ALK2 Mutations on BMP Pathway Signaling

The aforementioned studies[158,161,162] using FOP patient cells demonstrated elevated BMP pathway signaling in response to exogenous BMP ligand compared to normal cells, indicating that $ALK2^{R206H}$ has increased ligand-sensitivity. These investigations also revealed elevated canonical BMP signaling in the absence of BMP ligand, supporting that $ALK2^{R206H}$ is a mild gain-of-function mutation that remains ligand-responsive. To specifically demonstrate that *ALK2* mutations enhance BMP pathway signaling, several in vitro *ALK2* overexpression assays have been conducted. C2C12 cells (myoblast precursor cell line) overexpressing $ALK2^{R206H}$ showed increased phosphorylated SMAD1/5/8 levels, nuclear

localization of SMAD1/5/8 proteins, and enhanced *ID1* promoter activity,[163] all hallmarks of activated BMP pathway signaling.[23,94,164] Similar results were observed using COS-7 (monkey kidney cell line) and MC3T3-E1 cells (mouse preosteoblast cell line),[86,163] consistent with the results from patient cells, and demonstrating that the enhanced activity of $ALK2^{R206H}$ is not cell type specific.

Mouse embryonic fibroblasts (MEFs) can be isolated from $Acvr1^{R206H}$ mouse models[72,73] and used as an in vitro mesenchymal cell system to study elevated BMP pathway signaling conferred by the R206H mutation on a molecular level.[165] These cells are easier to obtain than patient cells, less heterogeneous in the expression of the mutation, and recapitulate the increased levels of SMAD1/5/8 phosphorylation and BMP target gene expression seen in patient LCL cells and SHED cells. These cells also can be differentiated to adipogenic, chondrogenic, and osteogenic lineages, making them amenable to study the effects of the FOP mutation.[165,166] Investigation of the FOP mutation utilizing this system demonstrated that the ALK2 receptor is necessary for the earliest stages for chondrogenesis and that at least one effect of ALK2 gain-of-function mutations in FOP patients is enhanced chondrogenic differentiation, which supports formation of heterotopic endochondral bone.[154,165,166]

BMP ligand independence of $ALK2^{R206H}$ was further demonstrated through genetic approaches using an in vivo BMP-null zebrafish model.[86] Early zebrafish embryos require both BMP2b and BMP7 for proper dorsal–ventral patterning.[84] However, excess BMP signaling induces ventralization in developing zebrafish embryos[83,84] providing an observable screening method for elevated BMP signaling. Zebrafish lacking both BMP2b and BMP7 exhibited moderate to severe ventralization following transfection of $ACVR1^{R206H}$ mRNA,[86] confirming that the BMP pathway is active in the absence of BMP ligand in vivo.

The observed gain-of-function activity of $ALK2^{R206H}$ is consistent with predictions based on structural homology modeling.[50,51] In addition to the pH-sensitive switch (see Section 5.1.1), the R206H mutation is also predicted to reduce binding of FKBP12, an inhibitor of TGFβ superfamily type I receptors that binds at the GS domain to prevent leaky activation of receptors in the absence of ligand.[50,167] Coimmunoprecipitation experiments revealed that in the absence of BMP ligand, FKBP12 shows reduced binding to $ALK2^{R206H}$ compared to wild type ALK2,[86] results that have been supported by other assays.[168,169] More extensive experiments using native gel electrophoresis and size exclusion high-performance liquid chromatography (HPLC) documented threefold decrease in binding of FKBP12 to $ALK2^{R206H}$, as compared to wild-type.[170] Structural homology investigation of the L196P nonclassical mutation also identified decreased receptor binding affinity for FKBP12.[51] This may contribute to enhanced receptor activity in the absence of ligand activation.

In addition to the classical R206H mutation in ACVR1 in FOP, BMP signaling assays of a limited number of FOP variant mutations have been reported.[51,63,171,172] Signaling assays in C2C12 cells transfected with either G356D mutation[171] or L196P mutation[172] showed that each variant receptor exhibited increased phosphorylated SMAD1/5/8 levels and enhanced *ID1* promoter activity in the absence of BMP ligand. This indicated enhanced receptor activity similar to the effects of the R206H mutation. Interestingly, a patient with L196P mutation had characteristic great toe malformations, but extremely mild progression of HO; in vitro cell signaling assays demonstrated signaling activity equal to the classic R206H mutation,[172] suggesting that the L196P and R206H mutations have similar embryonic developmental effects, but may function differently during HO. Caution in interpretation of these findings is warranted, as they are based on a single case. The Q207E variant mutation was initially predicted to function similarly to the engineered Q207D mutation, which results in irreversible relocation of the GS domain into an activating position. However, interestingly, the Q207E mutation was shown to act similarly to the classic R206H mutation and retain some ability to be inhibited by FKBP12.[63]

5.1.4 *Activin A Induction of TGFβ/BMP Signaling Pathway*

Activins are ligands that classically activate the TGFβ arm of the TGFβ/BMP superfamily signaling pathway, although have been also reported to bind to BMP type I and type II receptors during receptor complex formation.[173] Notably, activins are potent regulators of inflammation and participate in positive-feedback loops that potentiate expression of proinflammatory cytokines in many immune cell types.[174–178] Recently, Activin A was identified as a ligand that preferentially binds to $ALK2^{R206H}$, resulting in enhanced BMP signaling, demonstrated by an increase in phosphorylation of SMAD1/5/8.[72] This result was replicated using human-induced pluripotent stem cells obtained from FOP patients that were subsequently differentiated to mesenchymal stromal cells.[179] These results are notable because Activin A is normally associated with increased phosphorylation of SMAD2 and SMAD3, the downstream effectors of activated TGFβ signaling.

5.2 Effects of FOP ACVR1/ALK2 Mutation on Lesion Progression

Heterotopic lesion formation in FOP involves an initial catabolic phase of inflammation, tissue destruction, and turnover, followed by an anabolic tissue formation

and replacement phase.[9] Early lesions, most notably in children, begin with extensive soft tissue swelling that is associated with neutrophil, macrophage, mast cell, and lymphocyte infiltration.[22,24,180,181] Connective tissue degeneration follows immune cell infiltration; however, instead of the tissue regeneration that normally occurs in response to tissue injury, robust fibroproliferative areas composed of fibroblast-like cells form.[8,182] These fibroblast-like cells then differentiate to chondrocytes followed by osteogenesis leading to mature heterotopic bone. It is possible that the *ACVR1*^{R206H} mutation affects each stage of lesion development.

5.2.1 Immunological Contributions to Heterotopic Ossification Development in FOP

Flare-ups of HO in patients with FOP have been observed following inflammatory stimuli,[24] suggesting the participation of an inflammatory trigger in the development of HO. A comprehensive review of studies investigating immunological contributions to both genetic and nongenetic forms of HO was recently reported, highlighting the role of multiple immune cell types and signaling pathways in this process.[26] The BMP pathway also has a functional role in the immune system, and elevated BMP signaling resulting from ALK2^{R206H} may enhance an inflammatory response. For example, in response to BMP6, macrophages are induced to a proinflammatory state similar to the macrophage immune response induced by lipopolysaccharide.[183] BMP signaling also enhances thymocyte precursor survival and induce interferon gamma (IFN-γ) production in CD8-positive T cells.[184,185]

Recent studies have documented the importance of specific immune cell types in the development of HO in a variety of genetic and implant models of ectopic bone. The requirement of macrophages and mast cells for HO development has been investigated most frequently due to their robust presence in early and intermediate lesion stages of FOP.[73,181] Macrophages are present in the early FOP lesion[73] and have known roles in tissue damage clearance and remodeling, processes that may be aberrant in FOP. Increased mast cell numbers are observed at all stages of lesion development, with vast increases in mast cell density (upward of 40- to 150-fold) in FOP compared to unaffected individuals, suggesting an important function in the pathophysiology of this disease.[181]

The most direct evidence of macrophage and mast cell involvement in HO comes from studies in which these cells are selectively ablated or functionally inhibited in in vivo models. Ablation of macrophages via clodronate liposome-mediated[186] or diphtheria toxin-mediated-ablation[187] techniques in the *Nse-BMP4* mouse model of HO resulted in significantly reduced HO volume.[188] A mast cell deficient *Nse-BMP4*; *c-kit*$^{W\text{-}sh/W\text{-}sh}$ mouse model exhibited similar reductions of HO volume.[189]

Mast cell function in the development of HO is mediated through the proinflammatory neuropeptide Substance P (SP).[189] Elevated SP was detected in early HO lesions of patients with FOP and nonhereditary forms of HO, as well as in *Nse-BMP4* transgenic mice.[189] The source of SP was identified as the dorsal root ganglia. Inhibition of the SP receptor NK1r, or ablation of mast cells which express high levels of NK1r, greatly reduced the formation of HO in transgenic mice.[189] HO formation was also inhibited in mice lacking sensory neurons, which causes a significant decrease in SP and calcitonin gene related peptide (CGRP) levels.[190]

5.2.2 Role of Activin A in FOP Lesion Pathology

Given the recent discovery of Activin A preferentially binding to ALK2^{R206H}, resulting in enhanced BMP signaling, this factor is a prime candidate for investigation into effects on FOP lesion pathology and as a potential therapeutic target.[72] *Acvr1*$^{cR206H/+}$ mice treated with a humanized antibody against Activin A exhibited strongly reduced HO formation compared to controls, suggesting that Activin A is a key mediator in the development of HO in FOP.[72] Additional studies are necessary to investigate this newly discovered mechanism.

5.2.3 Role of Hypoxia in Heterotopic Ossification Pathology

Recent investigations have identified a link between the elevated BMP signaling present in FOP and activation of the hypoxia-sensing HIF1α pathway, contributing to HO development.[23] Inhibition of the HIF1α pathway by genetic or pharmacologic means restores canonical BMP signaling to normoxic levels in human FOP cells and profoundly reduced HO in a constitutively active *Acvr1*$^{Q207D/+}$ mouse model of FOP-like HO.[23] This is a notable development given that inhibition of the HIF1α pathway has been previously shown to prevent nongenetic and genetic incidents of HO.[191] This suggests that cellular oxygen-sensing mechanisms modulate BMP pathway signaling and contribute, in part, to HO development in FOP.[23,24,26,192]

5.2.4 Origin of Progenitor Cells Contributing to HO Formation

Identifying the origin of cells that differentiate to chondrocytes and osteoblasts during HO is an ongoing goal. Many cell types possess chondrogenic and osteogenic potential in vitro; however, far fewer cell types have been identified as participating in HO formation in vivo.[193] Although many candidate cells as sources for HO have been proposed, progress has been made in identifying at least a subset of cells that contributes to HO in FOP.

Mouse models of HO were used to identify Tie2 as a marker for approximately 50% of cells contributing to

HO and TIE2$^+$ cells were also identified in FOP patient lesion biopsies.[182,194] Subsequent studies suggest that Tie2$^+$ cells are directly influenced by expression of ALK2^{R206H} in response to activated BMP pathway signaling.[194] Endothelial cells transfected with ALK2^{R206H} showed morphological changes and induction of mesenchymal cell markers indicating that the cells acquired a dedifferentiated state through endothelial-to-mesenchymal transition (EndMT). These resulting mesenchymal-like cells can respond to inductive signals to differentiate into adipocytes, chondrocytes, or osteoblasts, demonstrating that the cell population is multipotent.[165,194] These data suggest that conversion of endothelial cells to mesenchymal stem-like cells could be an early anabolic event in the formation and progression of FOP-like lesions through a process that mimics normal skeletal development.[194] However, a Tie2$^+$ progenitor cell population of nonendothelial lineage with osteogenic potential has also been identified.[195] This mesenchymal cell population (Tie2$^+$, PDGFRα$^+$, SCA-1$^+$) is localized to the interstitium of skeletal muscle and other tissues.[195] Whether TIE2$^+$ endothelial and nonendothelial cells contribute to HO in vivo remains to be clarified.

5.2.5 Chondrogenesis and Osteogenesis

Many studies confirm that BMP pathway signaling, and in some cases ALK2 specifically, is a regulator of chondrogenesis and osteogenesis during endochondral ossification.[165,196–199] ALK2 is expressed in skeletal tissues and functions as a BMP type I receptor in both chondrocytes and osteoblasts in vitro.[196] Overexpression of constitutively active ALK2^{Q207D} enhances chondrogenic differentiation of chick embryonic fibroblasts (CEFs) in vitro, as well as cartilage expansion, delayed endochondral ossification, and joint fusion.[196] In mice, expression of a Cre-inducible *Alk2*^{Q207D} transgene in skeletal muscle induces endochondral heterotopic bone and joint fusion similar to observation in FOP patients.[115]

BMP signaling studies indicated that ALK2^{Q207D} exhibits a more robust constitutive activation of the BMP pathway compared to ALK2^{R206H}.[86,163,168,169] However, ALK2^{R206H} also significantly enhances chondrogenesis. Mouse embryonic fibroblasts (MEFs) expressing *Acvr1*^{R206H} exhibited accelerated chondrogenesis compared to wild type cells. Gene expression of early chondrogenic marker genes *Sox9*, *ColII* (Collagen type II), and *Acan* (Aggrecan) were upregulated in *Acvr1*^{R206H} MEFs.[165] This mirrored data from an overexpression model of the ALK2^{R206H} receptor in micromass and three-dimensional alginate bead chondrogenic cultures, which also showed increased expression of *ColII* and *Acan*.[86,165] By contrast, overexpression of ALK2^{Q207D} induced a dramatic increase of the late stage chondrogenic markers *Ihh* and *ColX*, while *Acan* expression is only slightly enhanced and *ColII* is significantly downregulated by ALK2^{Q207D}.[86] The BMP antagonist Noggin caused no inhibition of chondrogenesis induced by ALK2^{Q207D}; however, Noggin partially inhibited the enhanced differentiation caused by ALK2^{R206H}. These data demonstrated that the R206H mutation is mildly activating with BMP ligand independence. As predicted, the effect of ALK2^{R206H} was less robust compared to ALK2^{Q207D}; however, the in vitro chondrogenic assays previously described demonstrated that the ALK2^{R206H} mutation could enhance progenitor cell differentiation to cartilage in the context of the appropriate environmental and differentiation factors.

During heterotopic endochondral osteogenesis, hypertrophic chondrocytes provide a template for infiltrating osteoblasts. Given the profound effect of BMPs and BMP signaling in osteogenic differentiation and de novo bone formation, the effect of ALK2^{R206H} on the osteogenic potential of progenitor cells was evaluated using patient-derived SHED cells.[159] Cultured SHED cells from FOP patients show higher basal expression of the osteogenic markers *RUNX2* and *ALP* and also mineralize more rapidly than control SHED cells in the presence of osteogenic medium without BMP ligand. The enhanced BMP-independent osteogenic differentiation in addition to elevated *RUNX2* in vitro may "prime" SHED cells by accelerating differentiation to osteoblasts, consistent with the rapid induction of heterotopic bone in some FOP patients.[9,159] Human mesenchymal stem cells isolated from bone marrow and transduced with lentiviral *ACVR1*^{R206H} are similarly more sensitive to osteogenic differentiation.[169] The presence of ALK2^{R206H} increased synthesis of ALP and mineralization; however, this required the presence of BMP6 ligand.[169] Similar to chondrogenic differentiation experiments, ALK2^{R206H} showed a milder effect on osteogenesis when compared to ALK2^{Q207D}.[86,169]

The enhancing effect of ALK2^{R206H} on both chondrogenic and osteogenic differentiation in vitro supports that the mutation contributes to both of these steps of endochondral bone formation in FOP patients and is attributed directly to the enhanced BMP pathway signaling caused by the mutant receptor.

6 DIAGNOSTIC ASPECTS

The combination of great toe malformations with the appearance of rapid tumor-like swellings on the head, neck, and upper back of young children (Fig. 30.1) provides a diagnosis for FOP.[20,25] Clinical diagnosis of classic FOP can be confirmed by molecular diagnostics to determine the DNA sequence of the *ACVR1* gene and identify the *ACVR1*^{R206H} mutation. However, the clinical features of FOP are unique and distinct, and a diagnosis can be made through clinical evaluation alone, even prior to radiographic evidence of HO or *ACVR1* DNA sequence analysis.[25]

Clinical diagnosis of cases of FOP-plus and FOP variants are often more difficult, however, DNA sequencing can be especially useful to evaluate suspected cases of atypical FOP.

Given the extreme rarity of FOP, clinicians often fail to link the rapid appearance of soft tissue swellings with malformed great toes resulting in frequent misdiagnosis of FOP.[200] Common misdiagnoses are aggressive juvenile fibromatosis (extra abdominal desmoid tumors), lymphedema, and soft tissue sarcomas. Misdiagnosis has frequently resulted in unnecessary and harmful diagnostic biopsies that activate or exacerbate disease progression.[200] Biopsies carry high risk at any anatomic site, but are particularly life-threatening in the neck or back, where resulting asymmetric HO can lead to rapidly progressive spinal deformity and thoracic insufficiency syndrome, or in the jaw where ankylosis of the temporomandibular joints can lead to severe inanition.

A recent study reported that PET imaging detected an increased uptake of FDG (^{18}F-fluorodeoxyglucose) at sites of heterotopic bone formation in a patient with FOP.[201] FDG is taken up by metabolically active cells and may indicate the presence of early stage lesion formation suggesting the possibility of imaging as a diagnostic tool in the case of an uncertain clinical evaluation or to detect preosseous activity of HO.

7 COUNSELING AND TREATMENT

Flare-ups of FOP are sporadic and unpredictable, and there is great individual variability in the age of onset and rate of disease severity and progression.[3] Several large studies investigating the natural history of FOP have confirmed that it is impossible to predict the occurrence, duration, or severity of an FOP flare-up, although characteristic anatomic patterning has been described.[2,10] The rarity of FOP and the unpredictable nature of the condition make it extremely difficult to assess therapeutic interventions.

Presently, there are no effective medical or surgical options to prevent or reverse the formation of heterotopic bone in FOP. Although the heterotopic bone often forms as discrete, skeletal-like elements that would appear suitable for surgical resection, surgery is discouraged given that surgical removal of lesions is often followed by significant recurrence.[2,10,16] Surgical release of joint contractures has been unsuccessful and also risks new, trauma-induced HO.[16,41,200,202]

Currently, clinical management of FOP involves early diagnosis, prevention of trauma and other interventions that risk activating HO, and symptomatic treatment of pain associated with flare-ups.

Medical management is currently supportive.[202] The initial stages of heterotopic bone formation are associated with inflammation, and glucocorticoids seem effective in managing symptomatic new flare-ups affecting major joints of the appendicular skeleton, especially when used in early stages. Non-steroidal anti-inflammatory agents, cyclooxygenase-2 inhibitors, mast cell stabilizers, and leukotriene inhibitors are reported by patients to be effective in managing chronic pain and ongoing disease progression. These guidelines for symptomatic management of FOP are available through the International Fibrodysplasia Ossificans Progressiva Association (IFOPA) website (www.ifopa.org).

While physical therapy to maintain joint mobility may be harmful by provoking or exacerbating lesions, occupational therapy evaluations are often helpful in providing assistive devices for activities of daily living. Prevention of falls, influenza, recurrent pulmonary infections, and complications of restrictive chest wall disease is important. Intramuscular injections should be avoided.[202]

Oral health care should emphasize prophylaxis and should avoid injections for mandibular blocks and stretching of the jaw during dental procedures.[203] Dental techniques for focused administration of anesthetic without tissue trauma are available. Guidelines for general anesthesia in FOP have been reported.[203]

The most useful treatments for heterotopic bone formation would prevent, halt, or even reverse the progression of the condition. The prevention and treatment of HO in FOP, as well as approaches for treating more common forms of HO, will likely target multiple stages of the heterotopic bone formation process that could be used in combination therapies or specifically directed as warranted.

With emerging insights into the pathophysiology of ACVR1/ALK2-mediated HO, several strategies for the treatment and/or prevention of FOP have been proposed.[204] These approaches include blocking activity of the mutant FOP receptor and dysregulated BMP signaling pathway; inhibiting the inflammatory triggers and early-stage mediators of FOP flare-ups; altering the inductive and/or conducive microenvironments that promote the formation of FOP lesions; and diverting the responding chondro/osseous progenitors cells to a soft tissue fate.[204] The following sections will expand on a few of these promising approaches.

7.1 Blocking Activity of the Mutant Receptor in FOP

The identification of activating mutations in the *ACVR1* gene in FOP provided a specific target for drug development and intervention.[205] Plausible therapeutic approaches to inhibiting dysregulated ALK2 and BMP pathway signaling in FOP include monoclonal antibodies directed against ALK2 or ALK2 ligands, targeting the mutation through siRNA or gene-editing approaches,

small molecule inhibitors of ALK2 receptor activity, and soluble BMP antagonists.

Small molecule inhibitors are important molecular tools for interrogating signal transduction pathways with great potential as powerful therapeutic agents, and have been actively pursued as a treatment for FOP. The molecule dorsomorphin, identified in a screen for compounds that perturb dorsal–ventral axis formation in zebrafish,[85] has been recognized as an inhibitor of canonical BMP-SMAD signaling that blocked osteogenesis in vivo. Derivatives of dorsomorphin that specifically inhibit a subset of BMP type I receptors, with a particular emphasis on preferentially inhibiting ALK2, have been developed.[89,90,94,206] A safe and effective small molecule inhibitor for FOP will likely require inhibition of ALK2 preferentially over ALK3 and ALK6. Small molecule inhibitors for ALK2 with selectivity, efficacy, tolerance to resistance, lack of rebound effect, and acceptable safety profiles are greatly anticipated as a means of directly targeting the cause of FOP.

7.2 Altering the Cellular Microenvironment Supporting Heterotopic Ossification

Heterotopic bone formation occurs episodically, not continuously, in FOP patients; this indicates that while an activating *ACVR1* mutation is a required cause of the disease, the activity and consequences of the mutation can be modulated, allowing for periods of disease quiescence and activity and the opportunity to identify therapeutic interventions that target such modulators.

Episodes of FOP HO formation often occur following tissue injury, a finding suggests that trauma-associated conditions alter the connective tissue microenvironment and trigger events leading to ectopic bone formation. FOP flare-ups are associated with inflammation, a cause of tissue hypoxia,[207] and chondrogenesis and endochondral ossification are promoted by a hypoxic tissue environment. Hypoxia inducible factor one-alpha (HIF1α) is a key mediator of the cellular response to hypoxia and two recent studies established a regulatory role for HIF1α in HO, identifying a new target for prevention of HO and FOP.[23,191]

Stabilization of HIF1α occurs as an adaptive response to inflammation or hypoxia[207] and early inflammatory FOP lesions in humans[23] and in mouse models[23,191] are markedly hypoxic and express HIF1α. Inhibition of HIF1α by genetic or pharmacologic means reduces HEO in a constitutively active $Alk2^{Q207D}$ and trauma-induced mouse models of HO.[23,191]

Hypoxia prolongs the activation of cell surface protein kinase receptors by decelerating their endocytosis and degradation.[208] HIF1α was determined to increase the intensity and duration of BMP signaling through Rabaptin 5 (RABEP1)-mediated retention of ALK2 in the endosomal compartment of hypoxic connective tissue progenitor cells from patients with FOP.[23] Further, inhibition of HIF1α restores BMP pathway signaling to normoxic levels in human FOP cells.[23] Thus, an inflammation and cellular oxygen-sensing mechanism that modulates intracellular retention of a mutant BMP receptor determines, in part, its pathologic activity in FOP and identifying a previously unrecognized role of HIF1α in the hypoxic amplification of BMP pathway signaling and the induction HO.

7.3 Diverting Progenitor Cells From an Osseous to a Soft Tissue Fate

While targeting HO lesion formation at the earliest stages in the bone formation process has clear potential benefits, early events may not always be recognized resulting in a missed window of treatment opportunity. Shimono et al.[116] described an approach that blocks FOP-like heterotopic endochondral ossification after the events leading to bone formation have been initiated, providing an alternative or supplemental treatment strategy to early intervention. Retinoic acid is a potent inhibitor of chondrogenesis. This study demonstrated that retinoic acid receptor gamma (RARγ) mediates chondrogenesis and that RARγ agonists can block chondrogenesis preceding endochondral ossification.[116] Using in vivo heterotopic bone formation assays, RARγ agonists were found to effectively inhibit heterotopic endochondral ossification when used during a wide treatment window that included the prechondrogenic fibroproliferative phase, up to, but not including, the ossification phase.[116]

Activation of the RARγ pathway blocks not only BMP signaling through SMADs, but also impairs the differentiation potential of recruited progenitor cells by promoting proteosome-regulated degradation of BMP pathway–specific phosphorylated SMADs. Following discontinuation of RARγ agonist treatment, no significant rebound effect was observed,[116] indicating that the chondrogenic inhibition of RARγ signaling may be irreversible, perhaps through the mechanisms that redirect cell fate decisions in prechondrogenic mesenchymal progenitor cells to a nonosseous lineage.

One of the most effective RARγ agonists tested was palovarotene, a drug previously tested for respiratory conditions.[209] This potent RARγ agonist prevented injury-induced HO formation in multiple mouse models of HO, including a conditional $Acvr1^{cR206H/+}$ model expressing the classic FOP mutation.[71,116] While chondrogenesis is a known target of RARγ agonists, treatment of mice with global postnatal expression of $Acvr1^{cR206H/+}$ with palovarotene was found to also reduce the immune cell response following injury.[71]

Additionally, induction of $Acvr1^{R206H}$ expression during embryonic development, in skeletal progenitor

(*Prrx1*$^{+}$) cells, promoted noninjury induced HO, and palovarotene was effective in blocking this spontaneous HO.[71] This model also demonstrated that the FOP mutation impaired growth plate expansion in skeletal long bones resulting in a reduced hypertrophic zone and shorter bone lengths.[71] Treatment of control mice with palovarotene also reduced skeletal growth and impaired growth plate cartilage function, consistent with known effects of other retinoid agonists on skeletal growth. However, when *Prrx1-Acvr1*^{R206H} mice were treated with palovarotene, long bone growth was improved, growth plates were rescued to a near normal appearance, and the mice remained mobile and functional.[71]

The preclinical data identify RARγ agonists as a class of compounds that inhibit the BMP-induced chondrogenesis required for endochondral bone formation.[71,116] This approach targets the BMP signaling pathway during a specific pathological process of tissue metamorphosis that causes the most severe pathological consequences FOP. Palovarotene is currently being tested in an FDA-approved Phase 2 clinical trial for FOP (ClinicalTrials.gov identifier NCT02190747) to evaluate whether the drug will prevent HO development during and following flare-ups in FOP patients. Outcome measures include radiographic evaluation for HO presence, CT scan-assessed volume of developed HO, plasma biomarker levels, and joint range of motion. The ongoing Phase 2 clinical trial with palovarotene is one of the first trials to evaluate a compound directly targeting HO formation, and represents a significant milestone in the ongoing efforts to treat HO disorders.

8 CONCLUSIONS

FOP is a rare autosomal dominant disorder that is caused by gain-of-function mutations in the *ACVR1* gene. These mutations induce increased signaling by the BMP type I receptor ALK2 and cause the formation of progressive and extensive endochondral bone formation in extraskeletal connective tissues. Animal models of heterotopic bone formation, robust genetically-modified mouse models, along with in vitro assays will continue to provide important insight into the cellular and molecular mechanisms of cell differentiation and bone formation; and serve as platforms for developing and testing therapeutic strategies for FOP.

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CHAPTER

31

Thyroid Hormone in Bone and Joint Disorders

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1 INTRODUCTION

The requirement for thyroid hormones during linear growth and skeletal maturation has been recognized for over 125 years. In 1891, Von Recklinghausen reported a patient with thyrotoxicosis and multiple fractures, identifying the relationship between the thyroid and adult skeleton. During the last 25 years the role of thyroid hormones in bone and cartilage has attracted considerable attention, leading to important advances in the field.

2 THYROID HORMONE PHYSIOLOGY AND ACTION

2.1 Hypothalamic-Pituitary-Thyroid Axis

Synthesis and release of the prohormone 3,5,3′,5′-L-tetraiodothyronine (thyroxine, T4) and active hormone 3,5,3′-L-triiodothyronine (T3) are controlled by a negative feedback loop mediated by the hypothalamic-pituitary-thyroid (HPT) axis (Fig. 31.1A).[1] Thyrotropin releasing hormone (TRH) is secreted by the hypothalamus and acts on pituitary thyrotrophs to stimulate release of thyrotropin (thyroid-stimulating hormone, TSH). TSH acts via the TSH receptor (TSHR) on thyroid follicular cells to stimulate synthesis and secretion of T4 and T3. T3, derived mainly via local metabolism of T4, acts on thyroid hormone receptors α and β (TRα, TRβ) in the hypothalamus and pituitary to inhibit synthesis and secretion of thyrotropin releasing hormone and TSH. Normal euthyroid status is thus maintained by a negative feedback loop that establishes a physiological inverse relationship between TSH and circulating T3 and T4, and defines the HPT axis set point.

2.2 TSH Action

The glycoprotein hormone TSH is composed of α- and β-subunits and binds to the TSHR, a G-protein coupled transmembrane receptor (Fig. 31.1B). Although cAMP is the major second messenger following TSHR activation, alternative pathways have been implicated. In the thyroid the TSHR associates with various G proteins, and Gαs and Gαq are thought to compete for activation by the TSHR.[2] The TSHR has also been proposed to have diverse functions in extrathyroidal tissues, although their physiological significance has not been established. Thus, TSHR expression has been reported in anterior pituitary, brain, pars tuberalis, bone, orbital preadipocytes and fibroblasts, kidney, ovary and testis, skin and hair follicles, heart, adipose tissue, as well as hematopoietic and immune cells.[3]

2.3 Thyroid Hormone Transport

Cellular uptake of thyroid hormones is mediated by specific membrane transporters (Fig. 31.1C),[4] including monocarboxylate transporters MCT8 and MCT10, the organic anion transporter protein-1C1 (OATP1C1), and nonspecific L-type amino acid transporters 1 and 2 (LAT1, LAT2).[5] The best-characterized transporter MCT8 is expressed widely and its physiological importance has been demonstrated by inactivating mutations that cause the Allan–Herndon–Dudley X-linked psychomotor retardation syndrome (OMIM #300523).[6,7]

2.4 Thyroid Hormone Metabolism

T4 is derived from thyroid gland secretion, while the majority of circulating T3 is generated by deiodination of T4 in peripheral tissues. Although the circulating fT4

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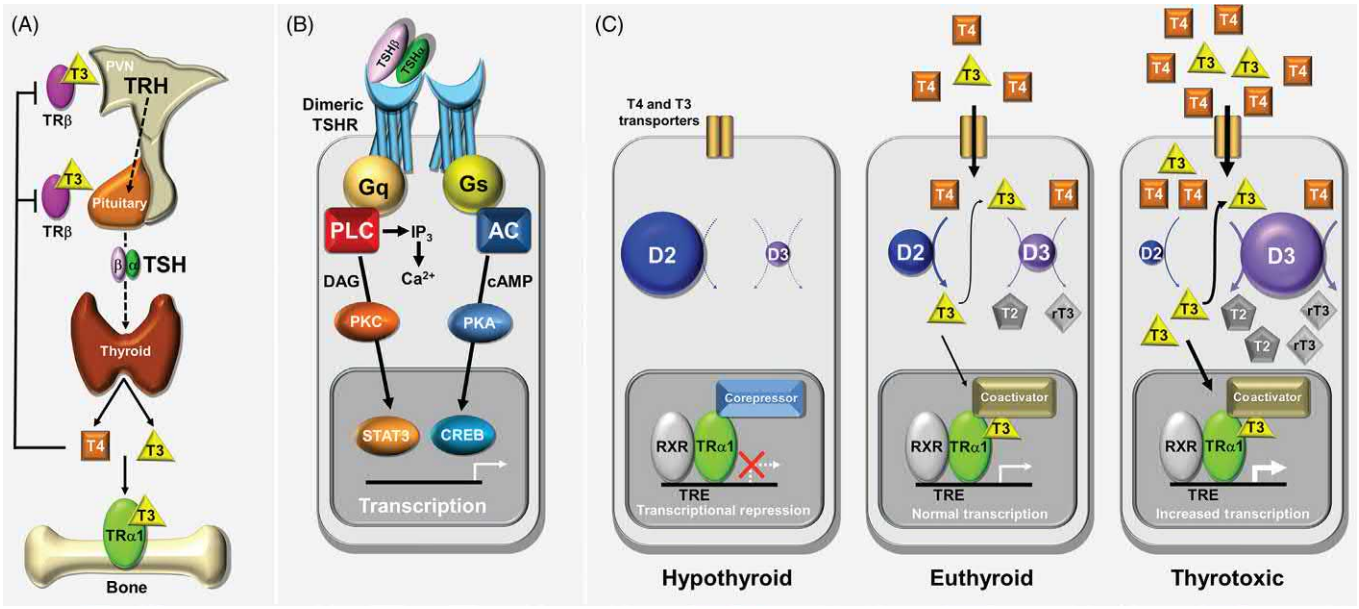

FIGURE 31.1 (A) Hypothalamic-pituitary-thyroid axis. The thyroid gland secretes the prohormone T4 and the active hormone T3 and circulating concentrations are regulated by a classical endocrine negative feedback loop that maintains an inverse physiological relationship between TSH and T4 and T3 (paraventricular nucleus, PVN). (B) TSH action. Binding of TSH to the TSHR results in activation of G protein coupled downstream signaling including the (1) adenylyl cyclase (AC), cAMP, protein kinase A (PKA), and the cAMP response element binding (CREB) protein, or (2) phospholipase C (PLC), inositol triphosphate (IP_3), and intracellular calcium pathway, or the (3) PLC, diacylglycerol (DAG), protein kinase C (PKC), and signal transducer and activator of transcription 3 (STAT3) pathway. (C) Thyroid hormone action in bone cells. In hypothyroidism, despite maximum DIO2 (D2) and minimum DIO3 (D3) activities, TRα1 remains unliganded and bound to corepressor thus inhibiting T3 target gene transcription. In the euthyroid state D2 and D3 activities are regulated to optimize ideal intracellular T3 availability resulting in displacement of corepressor and physiological transcriptional activity of TRα1. In thyrotoxicosis, despite maximum D3 and minimum D2 activities, supraphysiological intracellular T3 concentrations result in increased TRα1 activation and enhanced T3 target gene responses (reverse T3, rT3).

concentration is fourfold greater than fT3, the TR-binding affinity for T3 is 15-fold higher. Thus, T4 must be converted to T3 to mediate thyroid hormone action (Fig. 31.1C). Three iodothyronine deiodinases metabolize thyroid hormones. Type 1 deiodinase (DIO1) catalyses removal of inner or outer ring iodine atoms to generate T3, reverse T3 (rT3), or 3,3′-diiodothyronine (T2) depending on substrate. Most circulating T3 is derived from conversion of T4 to T3 by DIO1, which is expressed mainly in the thyroid gland, liver, and kidney. However, serum T3 concentrations are normal in *Dio1*$^{-/-}$ knockout mice and the physiological role of DIO1 is uncertain.[8] DIO2 generates T3 from T4 and its activity in skeletal muscle may also contribute to circulating T3, although the extent differs between species.[9,10] The major physiological role of DIO2 is to control the T3 concentration and saturation of the nuclear TR in target cells.[11] T4 treatment of cells in which MCT8 and DIO2 are coexpressed increases T3 target gene expression,[12] indicating thyroid hormone uptake and metabolism coordinately regulate T3 responsiveness. DIO3, by contrast, inactivates T3 or prevents T4 being activated by generating T2 or rT3, respectively. The physiological role of DIO3 is to limit access of thyroid hormones to specific tissues at critical times during development and in tissue repair.[13] The temporo-spatial and tissue-specific regulated expression of both DIO2 and DIO3 and the TRα and TRβ nuclear receptors provide a complex coordinated system for fine control of T3 availability and action in individual cell types.

2.5 Nuclear Actions of Thyroid Hormones

TRα and TRβ are ligand-inducible transcription factors that regulate T3-target gene expression (Fig. 31.1C). In mammals, *THRA* encodes three C-terminal variants: TRα1 is a functional receptor that binds DNA and T3, whereas TRα2 and TRα3 cannot bind T3 and act as antagonists in vitro.[14] An intron 7 promoter in mouse *Thra* gives rise to two variants, TRΔα1 and TRΔα2, which are potent dominant-negative antagonists in vitro, although their physiological role is unclear.[15] Two truncated TRα1 proteins p28 and p43 arise from alternate start codons and are proposed to mediate T3 actions in mitochondria or nongenomic responses.[16,17] *THRB* encodes two N-terminal TRβ variants, TRβ1 and TRβ2, both of which are functional receptors. Two further transcripts, TRβ3 and TRΔβ3, have been described but their physiological role is uncertain.[18] TRα1 and TRβ1 are expressed widely, but their relative concentrations differ during development and adulthood due to tissue-specific and temporo-spatial regulation,[19] so that most T3-target tissues are either predominantly TRα1 or TRβ1 responsive or lack isoform specificity.

In the nucleus, TRs form heterodimers with retinoid X receptors (RXR) and bind T3 response elements (TREs) in target gene promoters to regulate transcription. Unliganded TRs compete with T3-bound TRs for TREs and are potent repressors with critical roles during development. Unliganded TRs interact with corepressor proteins, which recruit histone deacetylases and inhibit gene transcription whereas T3-bound TRs interact with coactivators in a hormone-dependent fashion leading to target gene activation. Thus, opposing chromatin-modifying effects of unliganded and liganded TRs greatly enhance the magnitude of the transcriptional response to T3.[20] In addition to stimulatory effects, T3 also mediates transcriptional repression to inhibit key target genes, including TSH, although underlying mechanisms have not been characterized.

2.6 Nongenomic Actions of Thyroid Hormones

Nongenomic actions of thyroid hormones have been described at the plasma membrane, in the cytoplasm and in mitochondria but their physiological importance is unclear. The αVβ3 integrin mediates cell surface responses to T4 acting, for example, via the MAPK pathway to stimulate cell proliferation and angiogenesis.[21] TRβ also mediates rapid responses to T3, acting via the PI3K/AKT/mTOR/$p70^{S6K}$ and PI3K pathways,[22,23] whereas palmitolyated TRα activates the nitric oxide/protein kinase G2/Src pathway to stimulate MAPK and PI3K/AKT signaling in osteoblastic cells.[17]

3 ROLE OF THYROID HORMONES IN SKELETAL GROWTH AND DEVELOPMENT

3.1 Skeletal Cell Types

In early embryogenesis mesenchyme precursors condense and define a template for the future skeleton. These cells differentiate into chondrocytes that proliferate and secrete a matrix containing aggrecan and type II collagen to form a cartilage anlage or model of the skeletal element. Cells at the center of the anlage stop proliferating and differentiate into prehypertrophic and hypertrophic chondrocytes.[24] Hypertrophic chondrocytes increase rapidly in size, synthesize a matrix rich in type X collagen, and induce cartilage calcification before undergoing apoptosis.

Bone-forming osteoblasts comprise 5% of bone cells and derive from mesenchymal stem cells. Osteoblast maturation comprises precursor cell commitment, cell proliferation, type I collagen deposition, and matrix mineralization. Following bone formation, osteoblasts can differentiate into bone lining cells or osteocytes, or undergo apoptosis. In SOX9-expressing mesenchymal progenitors, osteoblastogenesis requires induction of RUNX2 and Osterix, and differentiation is regulated by the IHH, PTH, Notch, canonical Wnt, BMP, insulin-like growth factor-1 (IGF-1), and FGF-signaling pathways.[25]

Osteoclasts comprise 1%–2% of bone cells. They are multinucleated cells derived from fusion of mononuclear–myeloid precursors that resorb bone matrix and mineral. Attachment to bone is mediated by αVβ3 integrin that interacts with bone matrix proteins to form an actin ring and sealing zone, polarizing the osteoclast into ruffled border and basolateral membrane regions. An osteoclast-specific pump (H^+-ATPase) transports protons across the ruffled border and the acid environment dissolves hydroxyapatite while cathepsin K digests organic bone matrix. Commitment of hematopoietic stem cells to the myeloid lineage is regulated by the PU.1 and microophthalmia-associated transcription factors, which induce colony stimulating factor receptor (CSF-1R) expression. Macrophage colony stimulating factor/CSF-1R signaling stimulates expression of receptor activator of nuclear factor κB (RANK), leading to osteoclast precursor commitment. RANK ligand/RANK signaling induces nuclear factor κB (NFκB) and nuclear factor of activated T cells cytoplasmic 1 (NFATc1), leading to osteoclast differentiation and fusion.[25]

Osteocytes comprise 90%–95% of bone cells and derive from osteoblasts that have become embedded in bone matrix. Osteocyte dendritic processes ramify though networks of canaliculi and sense fluid shear stresses, communicating via gap junctions. Mechanical stresses and localized microdamage stimulate osteocytes to release cytokines and chemotactic signals, or induce apoptosis. In general, increased mechanical stress stimulates local osteoblastic bone formation, whereas reduced loading or microdamage results in osteoclastic bone resorption. Osteocytes control bone modeling and remodeling through their regulation of osteoclasts via the RANKL/RANK pathway and osteoblasts via modulation of Wnt signaling.[26]

3.2 Intramembranous and Endochondral Ossification

The flat bones of the face and skull form by intramembranous ossification. Mesenchyme progenitors within vascularized connective tissue membranes condense into nodules and differentiate to osteoblasts, which secrete an osteoid matrix of type I collagen and chondroitin sulfate that mineralizes to form an ossification center. The surrounding mesenchyme forms the periosteum and cells at the inner surface differentiate into lining osteoblasts. Progressive bone formation results in extension of bony spicules and fusion of adjacent ossification centers.[27]

Endochondral ossification is the process by which long bones form on a cartilage scaffold.[24] Mesenchyme precursors condense and differentiate into chondrocytes, which proliferate and secrete a matrix containing type II collagen and proteoglycans that forms a cartilage template. At the primary ossification center a coordinated program of chondrocyte proliferation, hypertrophic differentiation, and apoptosis leads to cartilage mineralization. Subsequently, vascular invasion and migration of osteoblasts enables replacement of mineralized cartilage with trabecular bone. Concurrently, perichondrial mesenchyme precursors differentiate into osteoblasts and form a collar of cortical bone. Secondary ossification centers form at the ends of long bones and remain separated from the primary ossification center by the epiphyseal growth plates where endochondral ossification continues.

3.3 Linear Growth and Bone Modeling

Growth plates at both ends of developing bones contain reserve, proliferative, prehypertrophic and hypertrophic zones.[24] The reserve zone contains uniform chondrocytes with a low proliferation index. Cells progress to the proliferative zone, become flattened and form longitudinal columns. As chondrocytes mature they express alkaline phosphatase, undergo terminal hypertrophic differentiation, secrete type X collagen, and increase in volume by 10-fold. Apoptosis of hypertrophic chondrocytes releases angiogenic factors that stimulate vascular invasion and migration of osteoblasts and osteoclasts, leading to remodeling of calcified cartilage and formation of trabecular bone. This ordered process mediates linear growth until adulthood.[24] Synchronously, the diameter of the long bone diaphysis increases by osteoblastic deposition of cortical bone beneath the periosteum, and the marrow cavity expands by osteoclastic bone resorption at the endosteal surface. Progression of endochondral ossification and linear growth is regulated by a local feedback loop involving IHH and PTHrP, and other factors including hormones, cytokines, and growth factors.[24] Linear growth continues until fusion of the growth plates during puberty, but bone mineralization and consolidation of bone mass continues until peak bone mass is achieved during the 3–4 decade.

3.4 Expression of TSH, TSHR, Thyroid Hormone Transporters, Deiodinases, and Thyroid Hormone Receptors in Skeletal Cells

TSHR expression in chondrocytes, osteoblasts, and osteoclasts suggests that TSH exerts direct actions in cartilage and bone.[28,29] TSHα and TSHβ subunits, however, are not expressed in osteoblasts or osteoclasts,[30,31] although an alternative splice variant of *Tshb* has been identified in mouse bone marrow and primary osteoblasts. Expression of this variant in bone marrow-derived macrophages activated cAMP in cocultured, stably transfected TSHR-overexpressing CHO cells.[32] The alternative TSHR ligand, thyrostimulin, is also expressed in osteoblasts and osteoclasts, and studies of *Gpb5*$^{-/-}$ mice lacking thyrostimulin indicated thyrostimulin regulates osteoblastic bone formation during early skeletal development.[31]

The thyroid hormone transporter MCT8 is expressed and regulated by thyroid status in growth plate chondrocytes, osteoblasts, and osteoclasts.[33,34] Recent studies indicate OATP1c1 is not expressed in the skeleton.[33] MCT10 appears to be the major transporter expressed in the growth plate,[35] while expression of LAT1 and LAT2 has also been detected in bone.[33–35] Nevertheless, the role and possible redundancy of thyroid hormone transporters in the skeleton has yet to be determined (Fig. 31.2).

Thyroid hormone metabolism occurs in skeletal cells.[36] Although DIO1 is not expressed in cartilage or bone,[34,36] the activating enzyme DIO2 is expressed in osteoblasts.[34,37] *Dio2* mRNA has also been detected in the embryonic mouse skeleton as early as embryonic day E14.5 and increases until E18.5.[38,39] In the developing chick growth plate, DIO2 activity is restricted to the perichondrium,[40] indicating the enzyme plays a role in regulation of fetal bone development. The inactivating DIO3 enzyme is present in all skeletal cell lineages particularly during development, with the highest activity in growth plate chondrocytes prior to weaning.[34,38] Thus, control of tissue T3 availability by DIO2 and DIO3 is likely to be important for skeletal development, linear growth, and osteoblast function (Fig. 31.2).

Both TRα1 and TRβ1 are expressed in bone but levels of TRα1 are at least 10-fold greater than TRβ1,[41] suggesting TRα1 is the predominant mediator of T3 action in bone. Nevertheless, other studies indicate TRβ may also play a role.[42] TRα1, TRα2, and TRβ1 are expressed in reserve and proliferative zone growth plate chondrocytes, immortalized osteoblastic cells, and in primary osteoblasts and bone marrow stromal cells. However, it is unknown whether TRs are expressed in osteocytes. Thyroid hormones stimulate osteoclastic bone resorption, but this effect may be indirect and mediated by T3-responsive osteoblasts.[3] Although immunolocalization of TR proteins and detection of TR mRNAs in osteoclasts from pathological human osteophytes and osteoclastoma tissue were reported in early studies, TR antibodies lack sufficient sensitivity to detect expression of endogenous protein and it remains uncertain whether osteoclasts express functional TRs or respond directly to T3. Overall, current studies indicate that reserve zone and proliferating chondrocytes, osteoblastic bone marrow stromal cells and osteoblasts are major

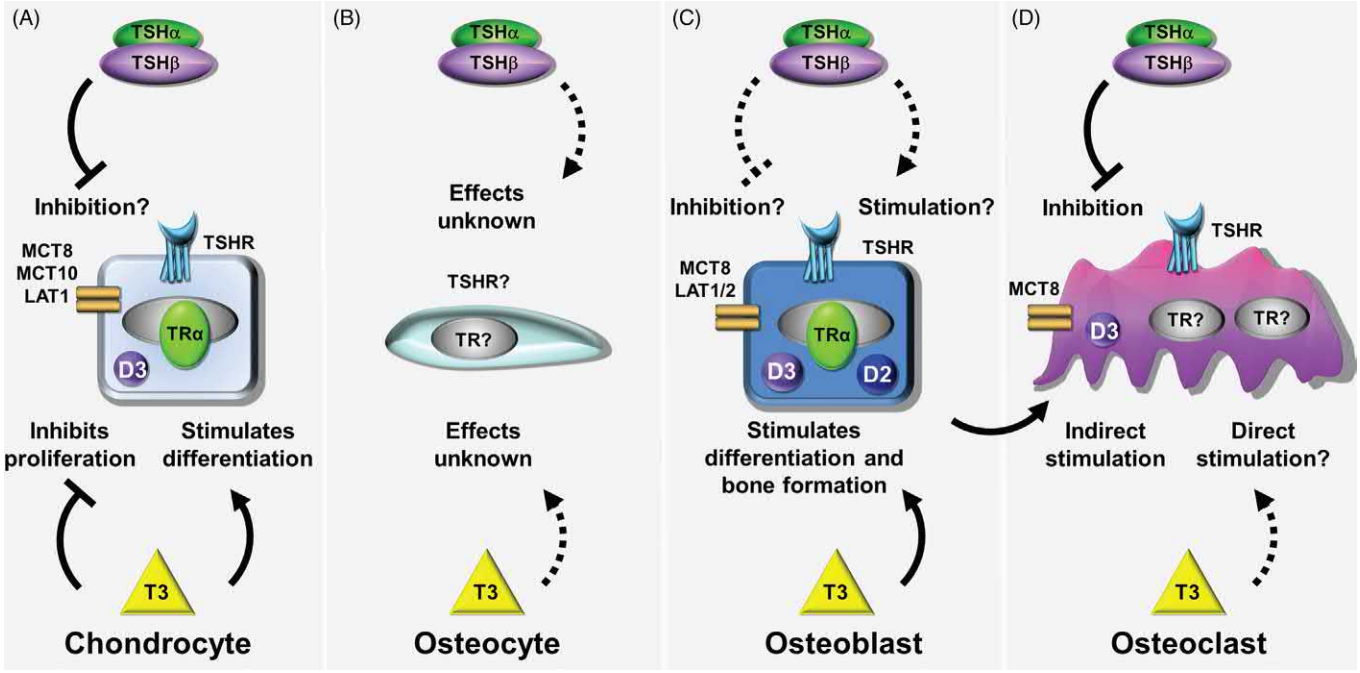

FIGURE 31.2 Actions of T3 and TSH in skeletal cells. (A) Chondrocytes express MCT8, MCT10, and LAT1 transporters, DIO3 (D3), TRs (predominantly TRα), and TSHR. T3 inhibits proliferation and stimulates prehypertrophic and hypertrophic chondrocyte differentiation, while TSH might inhibit proliferation and matrix synthesis. (B) T3 and TSH actions in osteocytes have not been investigated and it is unknown whether osteocytes express thyroid hormone transporters, deiodinases, TRs, or the TSHR. (C) Osteoblasts express MCT8 and LAT1/2 transporters, the DIO2 (D2) and D3, TRs (predominantly TRα), and TSHR. The majority of studies indicate T3 stimulates osteoblast differentiation and bone formation. Contradictory data suggest TSH may stimulate, inhibit, or have no effect on osteoblast differentiation and function. (D) Osteoclasts express MCT8, D3, TRs, and the TSHR. Currently it is unclear if T3 acts directly in osteoclasts or whether indirect effects in the osteoblast lineage mediate its actions. The majority of studies indicate TSH inhibits osteoclast differentiation and function.

T3-target cells in bone and predominantly express TRα (Fig. 31.2).

3.5 Pediatric Consequences of Thyroid Dysfunction

Hypothyroidism is the most common congenital endocrine disorder with an incidence of 1 in 1,800. Juvenile hypothyroidism results in delayed skeletal development and bone age with short stature. Delayed ossification and epiphyseal dysgenesis are evidenced by "stippled epiphyses" on X-rays. In severe or undiagnosed cases (Fig. 31.3) there is postnatal growth arrest and cessation of bone maturation with a complex skeletal dysplasia that includes broad flat nasal bridge, hypertelorism, broad face, patent fontanelles, scoliosis, vertebral immaturity with absence of ossification centers, and congenital hip dislocation.[43] Thyroid hormone replacement induces rapid "catch up" growth and accelerated skeletal maturation, demonstrating exquisite sensitivity of the developing skeleton to thyroid hormones. Nevertheless, predicted adult height may not be attained and final height deficit is related to the duration and severity of hypothyroidism prior to treatment. Overall, most children with congenital hypothyroidism that are treated early with thyroxine ultimately reach predicted adult height and achieve normal BMD.[44]

Graves' disease is the commonest cause of thyrotoxicosis in children but remains rare. Juvenile thyrotoxicosis is characterized by accelerated skeletal development and rapid growth, although advanced bone age results in early cessation of growth and persistent short stature due to premature fusion of the growth plates. In severe cases, early closure of the cranial sutures can result in craniosynostosis,[45] while maternal hyperthyroidism per se may also be a risk factor for craniosynostosis.[46]

3.6 Genetic Disorders of Thyroid Signaling

3.6.1 TSHB

3.6.1.1 Loss-of-Function Mutations

Several individuals have been described with mutations of *TSHB* leading to congenital nongoitrous hypothyroidism (OMIM #275100), but skeletal consequences have been documented in only two cases. Ten- and seven-year-old brothers had normal BMD following thyroid hormone replacement from birth,[47] demonstrating that absence of TSH throughout normal skeletal development

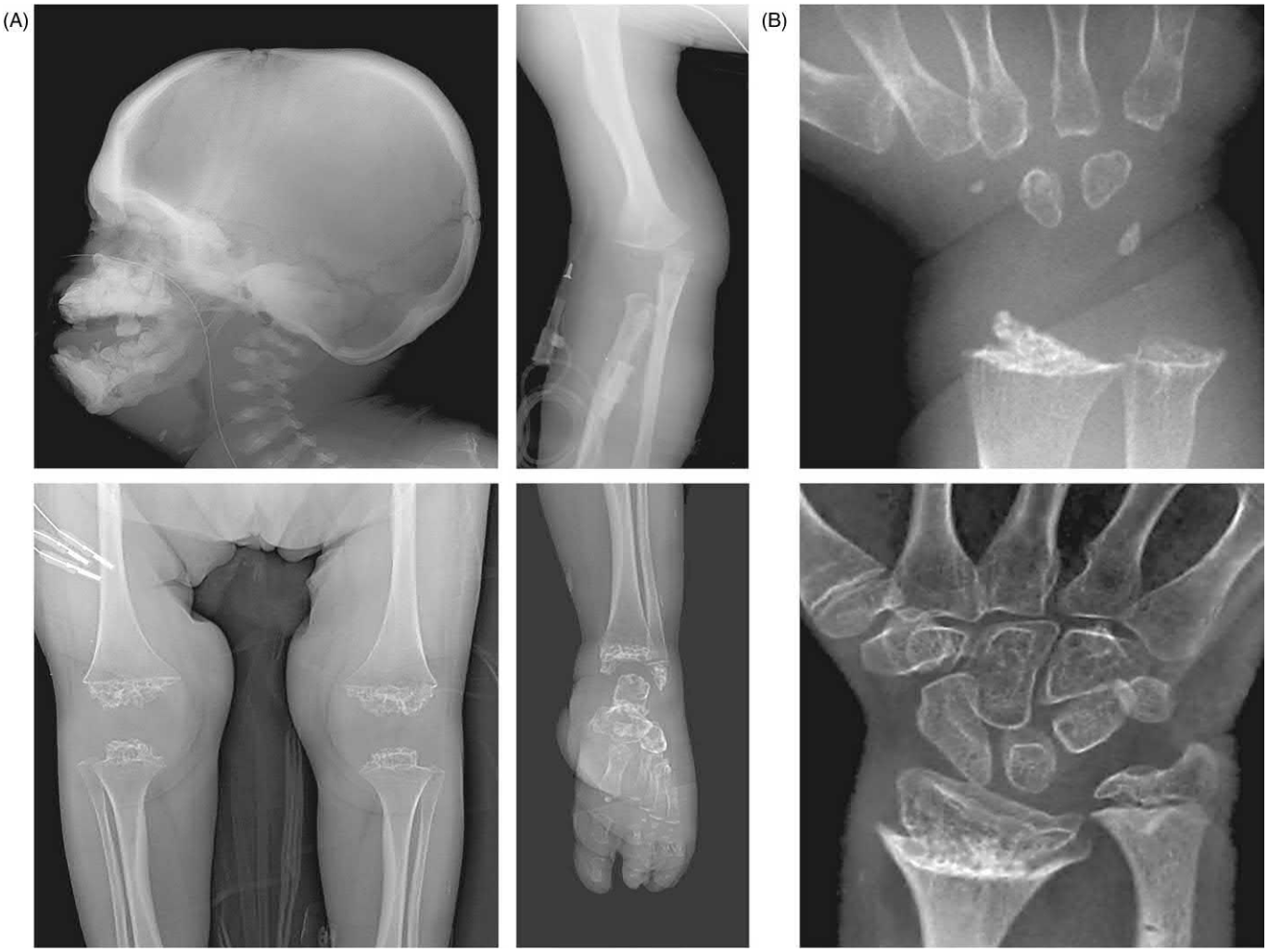

FIGURE 31.3 Radiographs of a 36-year-old woman with severe untreated congenital hypothyroidism. (A) Lateral skull image showing persistently patent sutures and fontanelles and delayed tooth eruption. Upper and lower limb radiographs showing severe epiphyseal dysgenesis with grossly delayed formation of secondary ossification centers. (B) Wrist radiographs showing a 35-year delay in bone maturation (bone age 14 months, upper panel), and bone age advanced by 8 years following 18 months of T4 replacement (lower panel), demonstrating rapid acceleration of endochondral ossification and "catch up growth." *Source: X-rays kindly provided by Dr. Jonathan LoPresti, Keck School of Medicine, University of Southern California.*

and growth does not affect bone mineral accumulation by 10 years of age.

3.6.2 TSHR

3.6.2.1 Loss-of-Function Mutations

Loss-of-function mutations at over 30 different amino acids result in varying degrees of TSH resistance and congenital nongoitrous hypothyroidism (OMIM #275200).[48] However, skeletal consequences have only been reported in a few children. Individuals with mild compensated hypothyroidism have normal growth and bone age, whereas subjects with severe thyroid hypoplasia have delayed intramembranous ossification and bone age at birth but normal growth and postnatal skeletal development following T4 replacement, indicating impaired TSHR signaling does not affect growth and bone maturation when thyroid hormones are adequately replaced.[3]

3.6.2.2 Gain-of-Function Mutations

Nonautoimmune autosomal dominant hyperthyroidism (OMIM #609152) is rare and patients are treated early with thyroid surgery and radioiodine ablation followed by T4 replacement. Skeletal manifestations at presentation include classical features of juvenile hyperthyroidism, with advanced bone age, craniosynostosis, and shortening of the fifth metacarpals and middle phalanges. In several younger patients amelioration of the phenotype was reported following treatment and normalization of thyroid hormones, indicating that early intervention improves skeletal abnormalities despite continued constitutive activation of the TSHR.[3]

3.6.3 SBP2

3.6.3.1 Loss-of-Function Mutations

The deiodinases are selenoproteins that require incorporation of the rare amino acid selenocysteine (Sec) for enzyme activity. Individuals with loss-of-function mutations of *SBP2*, which encodes the Sec incorporation sequence binding protein (SBP2) essential for incorporation of Sec, have abnormal thyroid hormone metabolism resulting in low circulating T3 levels despite elevated T4 and reverse T3 (OMIM #609698). Affected individuals have skeletal thyroid hormone deficiency with transient growth retardation, short stature, and delayed bone age.[49,50]

3.6.4 THRB

3.6.4.1 Dominant-Negative Mutations

Resistance to thyroid hormone (RTH) is an autosomal dominant condition caused by heterozygous dominant-negative mutations of *THRB* (OMIM #188570, RTHβ). The incidence of RTHβ is 1 in 40,000 and over 3,000 cases have been documented with 80% harboring *THRB* mutations, of which 27% are de novo.[51] The mutant TRβ disrupts the HPT axis resulting in elevation of T4 and T3, and inappropriately normal or increased TSH. There is a complex mixed phenotype of hyperthyroidism and hypothyroidism depending on the specific mutation and target tissue. Tissue responses to T3 are dependent upon: the severity of the TRβ mutation; genetic background; the relative concentrations of TRα, wild-type TRβ, and dominant negative mutant TRβ proteins in individual cells; and whether the patient has received prior treatment with antithyroid drugs or surgery. Consequently, interpretation of the skeletal phenotype in RTHβ is complex and various abnormalities have been described.

The skeletal consequences of RTHβ have been reported in a limited number of patients with a spectrum of mutations. Abnormalities include scaphocephaly, craniosynostosis, bird-like facies, vertebral anomalies, pigeon breast, winged scapulae, prominent pectoralis, and short 4th metacarpals. Some subjects have been reported with skeletal abnormalities similar to the consequences of hypothyroidism. Delayed growth was estimated in 20% of subjects,[52] while delayed bone age was reported in 29%–47%.[53] A study of 104 patients from 42 kindreds found short stature in 18%, low weight-for-height in 32%, and variable tissue resistance from kindred to kindred. RTHβ patients were shorter than unaffected family members and had no catch-up growth later in life. However, delayed bone age was documented in a minority.[54] In a study of 36 family members with RTHβ in four generations, delayed bone maturation with short stature was observed in affected adults and children. Of note, in affected individuals born to an affected mother, growth retardation was less marked. Seven of eight children with RTHβ, but only one of six controls, had bone age 2 SD below mean.[55] Delayed bone maturation was also found in a series of eight children.[56] In individual cases stippled epiphyses were reported in a 6-year-old,[57] intrauterine and postnatal growth retardation with delayed bone age in a 26-month-old girl,[58] and delayed bone age >3 SD below mean for chronological age in a 22-month-old.[59] Four patients with homozygous *THRB* mutations have been reported with markedly delayed linear growth and skeletal maturation.[3]

Other individuals with RTHβ have been reported with skeletal abnormalities similar to consequences of hyperthyroidism. Short metacarpals, metatarsals, and advanced bone age have all been described,[60] as well as craniofacial abnormalities, craniosynostosis, short stature, osteoporosis, and fracture. In 14 patients, including 8 adults and 6 children, affected children had short stature below the 3rd centile but no significant delay in bone age and adults had low BMD. Five adults from two unrelated kindreds with the same *THRB* mutation were followed for 3–11 years and found to have reduced BMD. In individual cases, a 15-year-old girl with short stature and advanced bone age was reported; craniosynostosis with frontal bossing and short metacarpals were seen in a 9-year-old; a 19-year-old man with increased osteocalcin and reduced BMD was reported; and a 21-year-old woman with short stature and elevated urinary deoxypyridinoline was described.[3]

Overall, these findings should be considered in the context of mouse models of RTHβ. In mutant mice, the phenotype is consistent with increased thyroid hormone action in bone manifest by accelerated skeletal development in juveniles and increased bone turnover with osteoporosis in adults.[61] The consistent findings in mice result from studies of single mutations in genetically homogeneous backgrounds that are not confounded by therapeutic intervention. Reports of human RTHβ result from patients with a broad range of mutations in diverse genetic backgrounds. Phenotype diversity is also likely to result from variable phenotype analyses and nomenclature among studies, retrospective and cross-sectional study design, differing surgical and pharmacological interventions, and analysis of individuals at differing ages.

3.6.5 THRA

3.6.5.1 Dominant-Negative Mutations

Heterozygous mutations of *THRA* (OMIM #614450, RTHα) were first reported in three families.[62–65] Affected individuals had grossly delayed skeletal development but variable motor and cognitive abnormalities. All had normal serum TSH with low/normal T4 and high/normal T3 concentrations, and a characteristically reduced fT4:fT3 ratio.

3.6.5.2 Mutations Affecting TRα1

A 6-year-old girl had skeletal dysplasia, growth retardation with grossly delayed bone age and tooth eruption, patent skull sutures, macrocephaly, flattened nasal bridge, disproportionate short stature, epiphyseal dysgenesis, and defective bone mineralization.[62] A 3-year-old girl displayed a similar phenotype and treatment with T4 resulted in a brief initial catch-up of growth, whereas growth hormone treatment had no effect. Her subsequent height, however, remained 2 SD below normal. Her 47-year-old father was short (−3.77 SD) but with normal BMD and had hearing loss due to otosclerosis.[64,65] A 45-year-old woman was subsequently described with disproportionate short stature and macrocephaly, together with skull vault and long-bone cortical thickening. Between the ages of 10 and 15 years, T4 treatment increased her growth, although final adult height was 2.34 SD below predicted.[63] Most recently, four individuals with truncating and missense mutations affecting TRα1 were described.[66] A consistent phenotype included disproportionate growth retardation with short limbs, hands and feet and a long thorax, and a skeletal dysplasia comprising flat nasal bridge with hypertelorism and macrocephaly. Radiological features included: ovoid immature vertebral bodies, ossification defects of lower thoracic and upper lumbar bodies, hypoplasia of the ilia and coxa vara. Hand X-rays showed short, wide, and deformed tubular bones. A phenotype–genotype correlation was noted with missense mutations associated with a milder phenotype. T4 treatment had no effect in any patient.[66]

3.6.5.3 Mutations Affecting Both TRα1 and α2

A 60-year-old woman presented in childhood with growth failure, macrocephaly, broad face, flattened nasal bridge, and a thickened calvarium.[67] Her growth improved after T4 treatment during childhood following a radiological opinion suggesting the skeletal features were similar to hypothyroidism. Her 30- and 26-year-old sons presented similarly and were also treated during childhood, resulting in a good growth response. Nevertheless, they each had a persistently abnormal facial appearance and macrocephaly, together with increased BMD between +0.8 and +1.9 SD. In vitro studies demonstrated the mutant TRα1 was a weak dominant-negative antagonist but its transcriptional activity could be restored by supraphysiological concentrations of T3, whereas the function of the mutant TRα2 did not differ from wild type.[67] Similarly, an 18-month-old girl presented with developmental delay and severe growth retardation and was found to have a missense mutation affecting both TRα1 and TRα2.[68] The mutant TRα1 had reduced T3 binding affinity and acted moderately as a dominant-negative, whereas the mutant TRα2 was indistinguishable from wild type. Twelve-month treatment with thyroxine resulted in catch-up growth although her height at 2.5 years remained 2.0SD below expected. The patient's untreated father exhibited typical features of macrocephaly and short stature, although BMD was normal.[68] Recently, a 27-year-old woman with skeletal dysplasia, low FT4:FT3 ratio, and a mutation affecting both TRα1 and α2 was described.[69] She had intrauterine growth retardation and failure to thrive, macrocephaly, hypertelorism, micrognathia, short and broad nose, clavicular and 12th rib agenesis, elongated thorax, ovoid vertebrae, scoliosis, congenital hip dislocation, short limbs, humeroradial synostosis, and syndactyly.[69] This atypical case extends the abnormalities described in patients with *THRA* mutations, and raises questions regarding whether TRα2 has a functional role in skeletal development or whether the RTHα phenotype is more diverse than previously reported.

3.7 TSH Action in the Growth Plate

Only limited information has been published regarding TSH action in cartilage. In mesenchymal stem cells, TSH stimulated chondrogenic gene expression suggesting TSH may increase chondrocyte differentiation.[70] However, growth plate cartilage and cultured chondrocytes express TSHR, and treatment with TSH increased cAMP activity and decreased expression of SOX9 and type IIa collagen expression.[29] Thus, the role of TSH in chondrocytes is uncertain (Fig. 31.2A).

3.8 Thyroid Hormone Action in the Growth Plate

Hypertrophic chondrocyte differentiation and vascular invasion of cartilage are sensitive to thyroid status, revealing the critical importance of T3 for endochondral ossification and linear growth. Nevertheless, studies of T3 action in chondrocyte monolayer cultures are conflicting due to the species, source of chondrocytes, and culture conditions. Consequently, three-dimensional systems have been devised to investigate the T3-regulated differentiation potential of chondrocytes in vitro.[3] T3 treatment of chondrogenic ATDC5 cells, mesenchymal stem cells, primary growth plate chondrocytes, and long bone organ cultures inhibits cell proliferation and concomitantly stimulates hypertrophic chondrocyte differentiation and cellular apoptosis.[71,72] T3 promotes hypertrophic differentiation by induction of cyclin-dependent kinase inhibitors to regulate the G1-S cell cycle checkpoint.[73] Subsequently, T3 stimulates BMP4 signaling, synthesis of a collagen X matrix, and expression of alkaline phosphatase and MMP13 to facilitate progression of hypertrophic differentiation and cartilage mineralization.[72,74] In addition, T3 regulation of growth plate chondrocyte

proliferation and differentiation in vitro involves activation of IGF-1 and Wnt signaling.[75]

The regulatory effects of T3 on endochondral ossification and linear growth in vivo involve interactions with key pathways that regulate growth plate maturation including IHH, PTHrP, IGF1, Wnt, BMPs, FGFs, and leptin.[3] IHH, PTHrP, and BMP receptor-1A participate in a negative feedback loop that promotes growth plate chondrocyte proliferation and inhibits differentiation thereby controlling the rate of linear growth. The set point of this feedback loop is sensitive to thyroid status[76] and is regulated by local thyroid hormone metabolism and T3 availability.[40] Furthermore, T3 stimulates expression of genes involved in cartilage matrix synthesis, mineralization, and degradation; including proteoglycans and collagen degrading enzymes, such as aggrecanase-2 (a disintegrin and metalloproteinase with thrombospondin motifs1, ADAMTS5) and MMP13, as well as BMP4, Wnt4, and FGFR3. In summary, thyroid hormone is essential for coordinated progression of endochondral ossification, stimulating genes that control chondrocyte maturation and cartilage matrix synthesis, mineralization, and degradation.[3]

4 ROLE OF THYROID HORMONES IN BONE MAINTENANCE AND MASS

4.1 Bone Remodeling Cycle

Functional integrity of the adult skeleton is maintained in a continuous process of repair by the "bone remodeling cycle." The basic multicellular unit of bone remodeling comprises osteoclasts and osteoblasts whose activities are orchestrated by osteocytes. Over 95% of the surface of the adult skeleton is normally quiescent because osteocytes exert resting inhibition of both osteoclastic bone resorption and osteoblastic bone formation.[77]

Under basal conditions, osteocytes secrete transforming growth factor-β (TGFβ) and sclerostin, which inhibit osteoclastogenesis and Wnt-activated osteoblastic bone formation, respectively. Increased load or local microdamage results in a fall in local TGFβ and recruitment of osteoclast progenitors. Osteocytes and bone lining cells express M-CSF and RANKL, the cytokines required for osteoclastogenesis. In addition to RANKL, osteoblasts and bone marrow stromal cells express osteoprotegerin (OPG). OPG is a secreted decoy receptor for RANKL and functions as the physiological inhibitor of RANK–RANKL signaling.[77] The RANKL:OPG ratio determines osteoclast differentiation and activity, and is regulated by systemic hormones and local cytokines.

Following the 30–40 day phase of bone resorption, reversal cells remove undigested matrix fragments from the bone surface and paracrine signals released from degraded matrix recruit osteoblasts and initiate bone formation. Over the next 150 days, osteoblasts secrete and mineralize new bone matrix (osteoid) to fill the resorption cavity. Although commitment of mesenchyme precursors to the osteoblast lineage requires both Wnt and BMP signaling, the canonical Wnt pathway acts as the master regulator of osteogenesis. During bone formation, some osteoblasts become embedded within newly formed bone and undergo terminal differentiation to osteocytes. Secretion of sclerostin and other Wnt inhibitors leads to cessation of bone formation and a return to the quiescent state in which osteoblasts become bone-lining cells.[78]

This cycle of targeted bone remodeling enables the adult skeleton to repair old or damaged bone, react to changes in mechanical stress, and respond to the demands of mineral homeostasis.

4.2 Clinical Consequences of Thyroid Dysfunction

Numerous studies have investigated the consequences of altered thyroid function on BMD and fracture risk but many are confounded by inclusion of subjects with thyroid disease and comparison of cohorts with combinations of pre- and postmenopausal women or men. Many studies lack statistical power because of small numbers, cross-sectional design or insufficient follow-up. Some studies measure TSH but not thyroid hormones, whereas others determine thyroid hormones but not TSH. Other confounders include inadequate control for: age; prior or family history of fracture; body mass index; physical activity; use of estrogens, glucocorticoids, bisphosphonates, or vitamin D; prior history of thyroid disease or use of thyroxine; and smoking or alcohol intake.[3] Different methods have also been used for skeletal assessment. Regulation of bone turnover has been investigated by histomorphometry, measurement of proinflammatory cytokines, and a variety of biochemical markers of bone formation [serum alkaline phosphatase, osteocalcin, carboxyterminal propeptide of type 1 collagen (P1NP)] and resorption [urinary pyridinoline and deoxypyridinoline collagen cross-links, hydroxyproline, carboxyterminal cross-linked telopeptide of type 1 collagen (CTX), cathepsin K], which are elevated in hyperthyroidism and generally correlate with disease severity.[3] Overall, hyperthyroidism shortens the bone remodeling cycle in favor of increased resorption and high bone turnover, but the duration of bone formation is reduced to a greater extent than the duration of bone resorption leading to a net loss of bone per remodeling cycle and osteoporosis. BMD has also been determined by several methods including dual X-ray absorptiometry (DXA), single-photon absorptiometry (SPA), dual-photon absorptiometry (DPA), quantitative computed

tomography (QCT), ultrasound, and high resolution peripheral QCT (HR-pQCT).

4.2.1 Discriminating Thyroid Hormone and TSH Effects on the Skeleton

Studies have been conflicting regarding the relative roles of thyroid hormone and TSH. Importantly, this issue cannot be resolved when the HPT-axis remains intact and the reciprocal relationship between thyroid hormones and TSH is maintained.[79] Nevertheless, simultaneously increased thyroid hormone and TSH signaling occurs in three clinical situations: nonautoimmune autosomal dominant hyperthyroidism and RTHβ, described earlier, together with Graves' disease, in which TSHR-stimulating antibodies persistently activate the TSHR and increase T4 and T3 production. Conventionally, secondary osteoporosis in Graves' disease is considered to result from elevated thyroid hormone levels and increased T3 actions in bone. By contrast, TSH has been proposed as a negative regulator of bone remodeling[28] and suppressed TSH levels in thyrotoxicosis have been suggested as the primary cause of bone loss. However, since Graves' disease is characterized by persistent autoantibody-mediated TSHR stimulation, patients should be protected. Despite this, Graves' disease is an established cause of secondary osteoporosis and fracture.

Acute effects of TSH on bone turnover have also been investigated. In patients receiving TSH-suppressive doses of T4 in the management of differentiated thyroid cancer, administration of recombinant human TSH (rhTSH) increases TSH levels but does not affect circulating T4 and T3 as patients have previously undergone thyroidectomy. In this context, treatment of premenopausal women with rhTSH had no effect on serum markers of bone turnover.[80–82] In postmenopausal women two studies reported a reduction in bone resorption markers accompanied by an increase in bone formation markers following rhTSH administration,[81,82] whereas two reported no effect.[80,83]

4.2.2 Consequences of Variation of Thyroid Status Within the Reference Range

A 6-year prospective study of 1278 healthy euthyroid postmenopausal women from 5 European cities (OPUS study) found that thyroid status at the upper end of the reference range was associated with lower BMD.[84] This finding is supported by cross-sectional and population studies from Europe, Asia, and the USA.[3]

Leader et al. investigated 13,325 healthy subjects and demonstrated an increased risk of hip fracture in euthyroid women, but not men, with TSH levels in the lower normal range.[85] Svare et al. analyzed prospective data from 16,000 women and 9,000 men in the Hunt2 study and found no overall relationship between baseline TSH and fracture, but weak positive associations in women between hip fracture risk and both low and high TSH.[86] In the OPUS study thyroid status at the upper end of the reference range was associated with an increased risk of incident nonvertebral fracture, whereas higher TSH was protective.[84]

Overall, thyroid status at the upper end of the euthyroid reference range is associated with lower BMD and an increased risk of fracture in postmenopausal women.

4.2.3 Consequences of Hypothyroidism

The effects of hypothyroidism on bone turnover have been investigated by histomorphometry. Manifestations include reduced osteoblast and osteoclast activities consistent with low bone turnover and a net increase in mineralization without major change in bone volume. Consistent with this, two studies reported normal BMD in patients newly diagnosed with hypothyroidism.[87,88] A cross-sectional cohort study of 49 patients with treated hypothyroidism studied using DXA, bone pQCT, and finite element analysis showed no difference between controls and patients receiving thyroxine.[89]

Nevertheless, large population studies identified an association between hypothyroidism and fracture.[88,90–93] In postmenopausal women a database study of 11,155 women over 65 receiving thyroxine revealed an increased risk of fracture in patients with a prior history of osteoporosis who were receiving more than 150 μg of T4, suggesting overtreatment is detrimental.[94] Overall, hypothyroidism is unlikely to be related to fracture, whereas long-term supraphysiological replacement may result in an increased fracture risk.

4.2.4 Consequences of Subclinical Hypothyroidism

A 12-year follow-up study of subclinical hypothyroidism in 4936 US men and women aged 65 years showed no association with BMD or incident hip fracture[95] and a 4.6 year-prospective study of men over 65 also showed no association between subclinical hypothyroidism and bone loss.[96] Although a 13-year-prospective study of 3,567 community-dwelling men over 65 revealed a 2.3-fold increased risk of hip fracture,[97] an individual participant metaanalysis of 70,298 individuals during 762,401 person-years follow-up found no association between subclinical hypothyroidism and fracture risk.[98]

4.2.5 Consequences of Subclinical Hyperthyroidism

Management of patients with differentiated thyroid cancer frequently involves prolonged treatment with T4 at doses that suppress TSH and may be detrimental to bone. Studies investigating the effect of TSH suppression on bone turnover have been inconsistent. Some reported increased bone formation and resorption markers, whereas others reported no effect.[3]

Most studies in premenopausal women reported no effect of TSH suppression on BMD, and no association

was identified in men.[95,96] Studies in postmenopausal women have been conflicting. For example, Franklyn et al. investigated 26 UK postmenopausal women treated for 8 years and found no effect on BMD,[99] whereas Kung et al. studied 46 postmenopausal Asian women and found decreased BMD.[100] Direct comparison between these studies is not possible, however, because TSH was fully suppressed in only 80% of patients in one,[99] mean calcium intake was low in the other,[100] while both were performed in small numbers of patients but from differing ethnic backgrounds. Similar conflicting data have been reported in less well-controlled cross-sectional and longitudinal studies.[3]

In a retrospective study of 2004 individuals, subclinical hyperthyroidism was associated with a 1.25-fold increased risk of fracture.[101] A case-control study of 213,511 individuals reported a 1.9-fold increased fracture risk in patients treated with T4 and demonstrated a dose response relationship.[102] A prospective study in postmenopausal women identified an association between suppressed TSH and fracture risk, with three- to fourfold increase in hip and vertebral fractures in individuals with TSH suppressed below 0.01 mU/L.[103] A 2.5-fold increased rate of hospital admission for fracture in subjects with TSH less than 0.05 mU/L,[91] and an exponential rise in the association between fracture risk and longer duration of TSH suppression[104] have also been reported. Further analysis demonstrated an increased fracture risk in patients with hypothyroidism that was strongly related to the cumulative duration of periods with a low TSH due to excessive thyroid hormone replacement.[105] Recently, Lee et al. demonstrated in a 14-year prospective study that men, but not women, with endogenous subclinical hyperthyroidism had a fivefold increased hazard ratio of hip fracture, while men with all causes of subclinical hyperthyroidism had a threefold increased hazard ratio.[97] Nevertheless, a prospective study in men aged 65 and older revealed no association between subclinical hyperthyroidism and fracture risk during 4.6 years follow-up, although a weak increased risk of hip fractures in subjects with lower serum TSH was identified.[96] No association between hip fracture risk and endogenous subclinical hyperthyroidism was seen in 4936 US men and women aged 65 years and older followed up for 12 years.[95]

In summary, large population studies have revealed increased bone turnover, reduced BMD, and an increased risk of fracture in postmenopausal women with subclinical hyperthyroidism. Although a recent systematic review and metaanalysis of seven population-based cohorts also suggested that subclinical hyperthyroidism may be associated with an increased risk of hip and nonspine fracture; however, a firm conclusion could not be reached due to limitations of the cohorts.[106] Subsequently, the largest metaanalysis of 70,298 individuals during 762,401 person-years of follow-up demonstrated an increased risk of hip and other fractures in individuals with subclinical hyperthyroidism, particularly in those with TSH suppressed below 0.1 mIU/L and those with endogenous disease.[98]

4.2.6 *Consequences of Hyperthyroidism*

Effects of thyrotoxicosis on bone turnover are consistent with histomorphometry data. Bone formation and resorption markers are elevated and correlate with disease severity in pre- and postmenopausal women and men. Severe osteoporosis due to uncontrolled thyrotoxicosis is rare because of prompt diagnosis and treatment, although undiagnosed hyperthyroidism is an important contributor to secondary bone loss and osteoporosis in patients presenting with fracture.[107] The presence of thyroid disease as a comorbidity factor has been suggested to increase 1- and 2-year mortality rates in elderly patients with hip fracture.[108] A metaanalysis of 20 studies of patients with thyrotoxicosis calculated that BMD was reduced at the time of diagnosis and there was an increased risk of hip fracture; antithyroid treatment returned the low BMD at diagnosis back to normal after 5 years.[109]

4.3 Human Population Studies

GWAS in osteoporosis cohorts have not identified associations between thyroid-related genes and BMD or fracture. Candidate gene studies investigated *TSHR*, *THRA*, and *DIO2*. The *TSHR* D727E polymorphism was associated with serum TSH and with osteoporosis diagnosed by qUS in 150 male subjects compared to 150 controls, whereas the D36H polymorphism was not.[110] By contrast, in 156 patients treated for differentiated thyroid cancer *TSHR* D727E was associated with higher femoral neck BMD independent of thyroid status, but this association was lost following adjustment for body mass index.[111] A similar association between *TSHR* D727E and increased femoral neck BMD was identified in subjects from the Rotterdam study.[112]

A candidate gene association study of 862 men over 65 investigated variation in *THRA* and BMD at the femoral neck and lumbar spine,[113] while the *THRA* locus was investigated in relation to BMD, fracture risk, and bone geometry in 27,326 individuals from the Genetic Factors for Osteoporosis (GEFOS) consortium and the Rotterdam Study 1 and 2 populations.[114] Both studies failed to identify relationships between bone parameters and genetic variation in *THRA*. In a further study, a cohort of 100 healthy euthyroid postmenopausal women with the highest BMD was selected from the OPUS population, and sequencing revealed no *THRA* abnormalities.[115] Yerges et al. identified an association between an intronic SNP in *THRB* and trabecular BMD in the MrOS study.[113]

Variation in deiodinases has been described as a potential genetic determinant of bone pathology. The *DIO2* T92A polymorphism was associated with decreased BMD and bone turnover markers in 154 patients treated for thyroid cancer.[116] However, in 641 young healthy men, no relationships between *DIO1* or *DIO2* variants and bone mass were identified.[117] Sequencing of *DIO2* in healthy postmenopausal women with high BMD also failed to identify abnormalities.[115]

4.4 TSH Action in Osteoblasts and Osteoclasts

TSHR expression has been documented in osteoblasts and osteoclasts,[28,30,31,118,119] but the lack of TSHα and β mRNA expression,[30,31] indicates TSH does not have autocrine effects in these cells. Nevertheless, treatment of osteoblasts with TSH in vitro inhibited osteoblastogenesis and differentiation.[28] By contrast, Sampath et al. and Baliram et al. showed that TSH stimulates osteoblast differentiation and function.[32,119] Furthermore, in ES cell cultures, TSH stimulated osteoblastic differentiation via protein kinase C and the noncanonical Wnt pathway.[120] In human SaOS2 osteosarcoma cells, TSH also stimulated proliferation and differentiation.[121] Finally, TSH stimulated osteoblast differentiation in stably transfected human osteoblastic U2OS-TSHR cells that overexpress the TSHR.[122] Despite these contrasting findings, Tsai et al. showed only low levels of TSHR expression, TSH binding and cAMP activation in human osteoblasts and concluded TSH was unlikely to have a physiological role.[123] Further studies also demonstrated low levels of TSHR protein in calvarial osteoblasts, and in these studies treatment with TSH and TSHR-stimulating antibodies failed to induce cAMP and TSH did not affect osteoblast differentiation or function.[30,118] Thus, TSH may inhibit, enhance, or have no effect on osteoblast differentiation and function (Fig. 31.2C).

Bassett et al. showed mouse osteoclasts express low levels of TSHR protein, but treatment with TSH and TSHR-stimulating antibodies did not stimulate cAMP or affect osteoclast differentiation and function.[30] Nevertheless, the majority of studies have shown TSH inhibits osteoclastogenesis and function.[28,118,119,124,125] Elevated levels of TNFα were proposed to mediate bone loss in *Tshr*$^{-/-}$ mice.[126] Nevertheless, mechanisms of bone loss appear complex and the underlying signaling pathways are incompletely defined. Thus, TSH inhibited osteoclastogenesis in WT mice and TNFα stimulated osteoclastogenesis in WT and *Tshr*$^{-/-}$ mice. Accordingly, TSH inhibited TNFα via AP-1 and RANKL-NFκB pathways in osteoclasts in vitro,[126] although in previous studies TSH was shown to stimulate TNFα in ES-cell derived osteoblasts.[120] Together, these findings were proposed as a counter-regulatory mechanism of TNFα inhibition and stimulation in osteoclasts and osteoblasts, respectively.[126] Overall, most studies indicate TSHR signaling inhibits osteoclastogenesis by complex mechanisms involving TNFα (Fig. 31.2D).

4.5 Thyroid Hormone Action in Osteoblasts and Osteoclasts

4.5.1 Osteoblasts

Although primary osteoblasts and osteoblastic cell lines respond to T3 in vitro, the consequences of T3 stimulation vary depending on species, anatomical origin of osteoblasts, cell type, passage number, confluence, stage of differentiation, and dose and duration of T3 treatment.[3] A general consensus indicates T3 stimulates osteoblast proliferation and differentiation and bone matrix synthesis, modification, and mineralization. T3 increases expression of osteocalcin, osteopontin, type I collagen, alkaline phosphatase, IGF-I and its regulatory binding proteins (IGF1BP-2 and -4), interleukin-6 and -8, MMP9, MMP13, tissue inhibitor of metalloproteinase-1 (TIMP-1), FGFR1 leading to activation of MAPK-signaling, and also regulates the Wnt pathway. Thus, T3 stimulates osteoblasts both directly and indirectly via complex pathways involving numerous growth factors and cytokines.[3]

Despite the many potential T3-target genes identified in osteoblasts, little mechanistic information is available.[3] T3 regulates osteoblastic cell morphology, cytoskeleton, and cell–cell contacts in vitro and stimulates osteocalcin via nongenomic actions mediated by suppression of Src. T3 also phosphorylates and activates p38 MAPK and stimulates osteocalcin expression in MC3T3 cells, a pathway that is enhanced by AMPK activation but inhibited by cAMP and Rho-kinase. A recent study further demonstrated that nongenomic signaling in osteoblasts and osteosarcoma cells is mediated by a plasma membrane bound N-terminal truncated palmitoylated isoform of TRα1. Acting via this isoform, T3 stimulated osteoblast proliferation and survival via increased intracellular Ca^{2+}, NO, and cGMP leading to activation of protein kinase GII, Src, and ERK.[17]

Overall, many studies in primary and immortalized osteoblastic cells demonstrate the complexities of T3 action in bone and emphasize the importance of the cellular system under study.

4.5.2 Osteoclasts

Osteoclasts express TRα1 and TRβ1 mRNAs but it is not clear whether functional receptors are expressed because TR antibodies lack sensitivity to detect endogenous proteins. Studies of mixed cultures containing osteoclast lineage cells and bone marrow stromal cells have been contradictory and it is unclear whether stimulation of bone resorption results from direct T3-actions

in osteoclasts or indirect effects in cells of the osteoblast lineage. Studies of fetal long bone and calvarial cultures implicated various cytokines and growth factors including IGF-1, prostaglandins, interleukins, TGFβ, and interferon-γ as mediators of secondary responses in osteoclasts.[3] Similarly, treatment of immortalized osteoblasts or primary stromal cells resulted in increased RANKL, interleukin 6 (IL-6), IL-8 and prostaglandin E2 expression, and inhibition of OPG, consistent with an indirect effect of thyroid hormones on osteoclast function. Other studies suggest that T3 effects on osteoclastogenesis are independent of RANKL signaling.[3]

Overall, it is unclear whether T3 acts directly in the osteoclast lineage, or whether its stimulatory effects on osteoclastogenesis and bone resorption are secondary responses to direct actions in other cell lineages.

5 GENETICALLY MODIFIED MICE

5.1 Targeting TSHR Signaling

5.1.1 Skeletal Development and Growth

TSHR knockout (*Tshr*$^{-/-}$) mice have congenital hypothyroidism with undetectable thyroid hormones and 500-fold elevation of TSH, are growth retarded, and usually die at weaning. Nevertheless, animals supplemented with thyroid extract regain weight by 7 weeks. Heterozygous *Tshr*$^{+/-}$ mice are euthyroid with normal linear growth. Untreated *Tshr*$^{-/-}$ mice had reduced BMD with increased bone formation and resorption when analyzed during growth at 6 weeks of age. *Tshr*$^{-/-}$ mice treated with thyroid extract displayed a 20% reduction in BMD and reduced calvarial thickness, although histomorphometry responses were not reported.[28] Heterozygotes had a 6% reduction in total BMD, affecting only some skeletal elements, no change in calvarial thickness and no difference in bone resorption or formation parameters. TSH was thus proposed as an inhibitor of bone formation and resorption.[28] Normally, T4 and T3 levels rise rapidly to a physiological peak at 2 weeks of age in mice, when growth velocity is maximal.[127] Since *Tshr*$^{-/-}$ mice are only supplemented with thyroid extract from weaning, they remain grossly hypothyroid at this critical stage of skeletal development. Thus, the phenotype reported in *Tshr*$^{-/-}$ mice also reflects the effects of severe hypothyroidism followed by incomplete "catch-up" growth and accelerated bone maturation in response to delayed thyroid hormone replacement.[3] Furthermore, treatment with supraphysiological doses of T4 for 21 days resulted in increased bone resorption and a greater loss of bone in *Tshr*$^{-/-}$ mice compared to wild-type controls, suggesting *Tshr* deficiency exacerbates bone loss in thyrotoxicosis.[128]

To investigate the relative importance of T3 and TSH in bone development, two contrasting mouse models of congenital hypothyroidism were compared, in which the reciprocal relationship between thyroid hormones and TSH was either intact or disrupted.[30] *Pax8*$^{-/-}$ mice lack a transcription factor required for thyroid follicular cell development and hyt/hyt mice harbor a loss-of-function mutation in *Tshr*. *Pax8*$^{-/-}$ mice have a 2000-fold elevation of TSH[129] and a normal TSHR, whereas hyt/hyt mice have a 2000-fold elevation of TSH but a nonfunctional TSHR. Thus, if TSHR has the predominant role these mice should display opposing skeletal phenotypes. However, *Pax8*$^{-/-}$ and hyt/hyt mice each display characteristic features of juvenile hypothyroidism.[30] Nevertheless, the actions of thyroid hormone and TSH are not mutually exclusive, and the consequences of grossly abnormal thyroid hormone levels in *Pax8*$^{-/-}$ and hyt/hyt mice may mask effects of TSH on the skeleton.

Gpb5$^{-/-}$ mice lacking the high-affinity ligand thyrostimulin were characterized to investigate further the role of TSHR in bone. Juvenile *Gpb5*$^{-/-}$ mice had increased bone volume and mineralization due to increased osteoblastic bone formation, whereas no effects on linear growth or osteoclast function were identified. Resolution of these abnormalities by adulthood was consistent with transient postnatal expression of thyrostimulin in bone.[31] Treatment of osteoblasts with thyrostimulin in vitro had no effect on cell proliferation, differentiation, and signaling, suggesting thyrostimulin inhibits bone formation indirectly during skeletal development.

5.1.2 Adult Bone Maintenance

Ovariectomized rats were treated with TSH at intermittent doses insufficient to alter circulating T3, T4, or TSH levels.[119] Treatment reduced bone resorption and increased formation, resulting in increased BMD. This osteoblastic response to TSH[119] contrasts with findings in *Tshr*$^{-/-}$ mice, which also displayed increased osteoblastic bone formation despite the absence of TSHR signaling.[28] In subsequent studies, intermittent treatment of ovariectomized rats or mice with similar concentrations of TSH prevented bone loss and increased bone mass following ovariectomy.[130] Furthermore, treatment of thyroidectomized and parathyroidectomized rats with intermittent TSH also suppressed bone resorption and stimulated bone formation resulting in increased bone volume and strength.[131]

5.2 Targeting Thyroid Hormone Transport and Metabolism

5.2.1 Thyroid Hormone Transporters

Mct8$^{-/y}$ knockout mice have elevated T3 and decreased T4 levels, recapitulating the thyroid abnormalities observed in Allan–Herndon–Dudley syndrome. *Mct8*$^{-/y}$ mice, however, do not display neurological abnormalities and exhibit only minor growth delay,

suggesting other transporters compensate for lack of MCT8 in mice.[132] *Mct10* mutant mice with an ENU loss-of-function mutation gain weight normally and mice lacking both MCT8 and MCT10 also display no evidence of growth retardation,[133] suggesting these transporters are dispensable. Nevertheless, although *Oatp1c1*$^{-/-}$ knockout mice gain weight normally, double mutants lacking *Mct8* and *Oatp1c1* exhibit growth retardation,[134] confirming redundancy among thyroid hormone transporters in regulation of skeletal growth. Finally, mice lacking *Mct8* and *Dio1* or *Dio2* display mild growth retardation, while triple mutants lacking *Mct8*, *Dio1*, and *Dio2* exhibit more severe growth delay,[132] indicating cooperation between thyroid hormone transport and metabolism during growth.

5.2.2 *Deiodinases*

A transient impairment of weight gain was reported in male *Dio2*$^{-/-}$ mice, whereas weight gain and growth were normal in *Dio1*$^{-/-}$ and DIO1-deficient C3H/HeJ mice, and in C3H/HeJ/*Dio2*$^{-/-}$ mutants with DIO1 and DIO2 deficiency.[135–137]

The role of DIO2 was investigated further in *Dio2*$^{-/-}$ mice,[37] which have mild pituitary resistance to T4 small increases in TSH and T4 but normal T3 concentrations. Bone formation and linear growth were normal in *Dio2*$^{-/-}$ mice, indicating DIO2 does not have a major role during postnatal skeletal development. This is unexpected given studies in the chick embryonic growth plate indicating DIO2 regulates the pace of chondrocyte proliferation and differentiation during early development.[40] Nevertheless, adult *Dio2*$^{-/-}$ mice have a generalized increase in bone mineralization and brittle bones resulting from reduced bone formation and decreased T3 signaling in osteoblasts.[37]

Dio3$^{-/-}$ mice have severe growth retardation and increased perinatal mortality. At weaning they have markedly reduced in body weight, which persists into adulthood.[138] The phenotype, however, is complicated by systemic effects of disrupted HPT axis maturation and altered thyroid status.

In summary, DIO1 has no role in the skeleton; DIO2 is essential for osteoblast function and maintenance of adult bone structure and strength, while the role of DIO3 in bone remains unclear.

5.3 Targeting TRα (Fig. 31.4)

Analysis of TR-null, Pax8-null, and TR-"knock-in" mice has provided insight into the relative roles of TR isoforms. TRα1$^{-/-}$ and TRα2$^{-/-}$ mice have deletion of TRα1 or α2, TRα$^{-/-}$ mice represent an incomplete deletion because the TRΔα1 and TRΔα2 isoforms are still expressed, whereas TRα$^{0/0}$ mice have complete loss of all *Thra* transcripts.[139–141]

TRα1$^{-/-}$ mice retain normal TRα2 expression, have mild central hypothyroidism but normal linear growth and weight gain.[142] TRα1$^{-/-}$TRβ$^{-/-}$ double-null mice have 60-fold increase in T4 and T3 with 160-fold higher TSH and decreased GH and IGF-1. Juveniles had growth retardation, delayed endochondral ossification, and decreased bone mineralization, and adults had reduced trabecular and cortical BMD.[143,144]

Gene targeting to prevent TRα2 expression resulted in 3–5-fold and 6–10-fold overexpression of TRα1 in TRα2$^{+/-}$ and TRα2$^{-/-}$ mice, respectively.[141] TRα2$^{-/-}$ mice had mild thyroid dysfunction and juveniles had normal linear growth, whereas adults had reduced bone mass.[141] Fusion of green fluorescent protein (GFP) to exon 9 of *Thra* in TRα1-GFP mice unexpectedly resulted in loss of TRα2 expression in homozygotes with only a 2.5-fold increase in TRα1 mRNA.[145] Homozygous TRα1-GFP mice were euthyroid with no abnormalities of postnatal development or growth, suggesting the phenotype in TRα2$^{-/-}$ mice may result from abnormal overexpression of TRα1. TRα2$^{-/-}$TRβ$^{-/-}$ double mutants have mild hypothyroidism resulting in transiently delayed weight gain.[146]

TRα$^{-/-}$ mice are markedly hypothyroid, have severely delayed bone development and die around weaning unless treated with T3.[147,148] TRα$^{-/-}$TRβ$^{-/-}$ double-null mice have 10-fold increase in T4 and T3 with 100-fold elevation of TSH and similarly display delayed endochondral ossification and growth retardation.[147,148]

TRα$^{0/0}$ mice are euthyroid and have less severe skeletal abnormalities than TRα$^{-/-}$ mutants. Juveniles display growth retardation, delayed endochondral ossification, and reduced bone mineral deposition, whereas adults had increased bone mass resulting from a bone-remodeling defect.[149] Deletion of both TRs in TRα$^{0/0}$TRβ$^{-/-}$ mice resulted in a severe phenotype of delayed bone maturation that may be due to reduced GH/IGF-I levels or reflect partial TRβ compensation in TRα$^{0/0}$ single mutants.[150]

The role of unliganded TRs in bone was studied in *Pax8*$^{-/-}$ mice. The severely delayed endochondral ossification in *Pax8*$^{-/-}$ mice compared to TRα$^{0/0}$TRβ$^{-/-}$ double mutants indicates the presence of unliganded receptors is more detrimental to skeletal development than TR deficiency. Importantly, amelioration of the *Pax8*$^{-/-}$ skeletal phenotype in *Pax8*$^{-/-}$TRα$^{0/0}$ mice, but not *Pax8*$^{-/-}$TRβ$^{-/-}$ mutants,[151] suggested that unliganded TRα is largely responsible for severity of the *Pax8*$^{-/-}$ phenotype. Nevertheless, this interpretation was not supported by analysis of *Pax8*$^{-/-}$TRα1$^{-/-}$ mice in which growth retardation in *Pax8*$^{-/-}$ mice was not ameliorated by additional deletion of TRα1.[152]

The essential role for TRα in bone was confirmed by studies of mice with dominant negative mutations of *Thra1*. A patient with severe RTH was found to have a

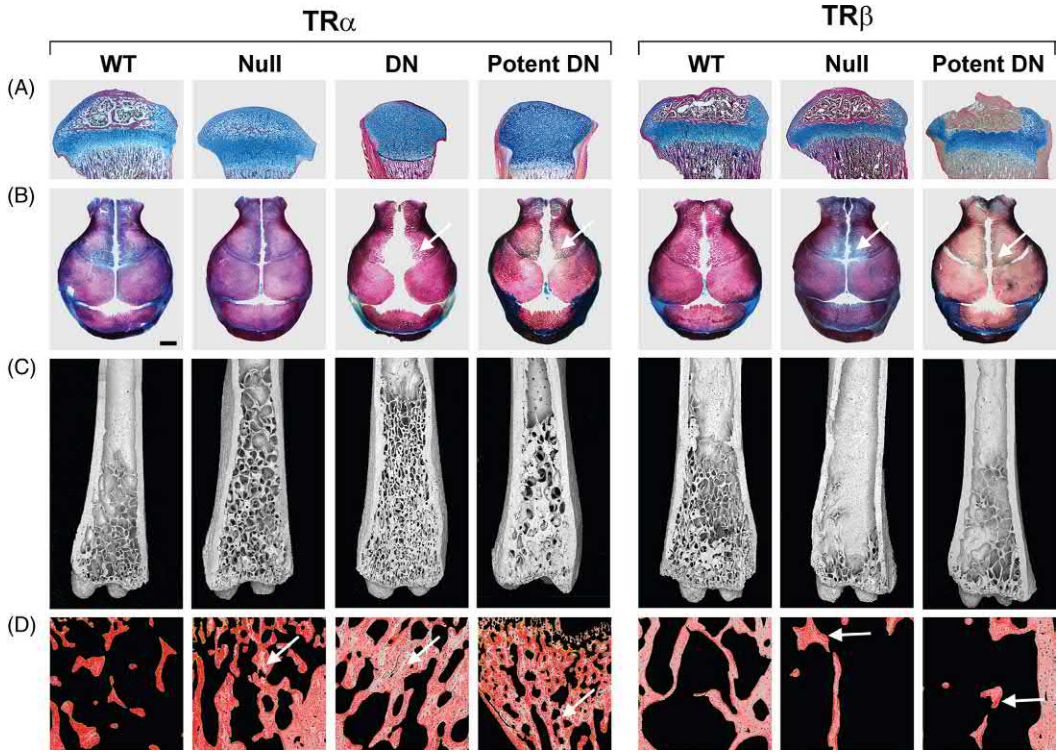

FIGURE 31.4 Skeletal phenotype of TRα and TRβ mutant mice. (A) Proximal tibias stained with alcian blue (cartilage) and van Gieson (bone) showing delayed formation of the secondary ossification center in TRα deficient mice ($TR\alpha^{0/0}$) and grossly delayed formation in mice with dominant negative TRα mutations ($TR\alpha1^{R384C/+}$ and $TR\alpha1^{PV/+}$). Mice with mutation or deletion of TRβ have advanced ossification with premature growth plate narrowing. (B) Skull vaults stained with alizarin red (bone) and alcian blue (cartilage) showing skull sutures and fontanelles. Arrows indicate delayed intramembranous ossification in mice with dominant negative TRα mutations ($TR\alpha1^{R384C/+}$ and $TR\alpha1^{PV/+}$) and advanced ossification in mice with mutation or deletion of TRβ. (C) Trabecular bone microarchitecture in adult TR mutant mice. Backscattered electron scanning electron microscopy images show increased trabecular bone in $TR\alpha^{0/0}$ mice and severe osteosclerosis in $TR\alpha1^{R384C/+}$ and $TR\alpha1^{PV/+}$ mice. By contrast, TRβ mutant mice have reduced trabecular bone volume and osteoporosis. (D) Trabecular bone micromineralization in adult TR mutant mice. Pseudocolored quantitative backscattered electron scanning electron microscopy images showing mineralization densities in which high mineralization density is represented by darker shading and low mineralization by lighter shading. Mice with deletion or mutation of TRα have retention of highly mineralized calcified cartilage *(arrows)* demonstrating a persistent remodeling defect. By contrast, mice with deletion or mutation of TRβ have reduced bone mineralization *(arrow)* secondary to increased bone turnover.

frameshift mutation in the *THRB* gene, termed "PV." The mutation results in expression of a mutant TR that cannot bind T3 and acts as a potent dominant-negative antagonist of wild-type TR function. The homologous mutation was used to generate $TR\alpha1^{PV}$ mice. The $TR\alpha1^{PV/PV}$ homozygous mutation is lethal whereas $TR\alpha1^{PV/+}$ heterozygotes display mild thyroid failure with small increases in TSH and T3, but no change in T4. $TR\alpha1^{PV/+}$ mice have severe growth retardation, delayed intramembranous and endochondral ossification, impaired chondrocyte differentiation, and reduced mineral deposition during growth.[61,127] Adult mice had grossly abnormal skeletal morphology with increased bone mass and defective bone remodeling.[153] Thus, $TR\alpha1^{PV/+}$ mice have markedly impaired bone development and maintenance despite systemic euthyroidism, indicating that wild-type TRα signaling in bone is impaired by the dominant-negative PV mutation in heterozygous mice. Notably, the presence of a dominant-negative TRα leads to a more severe phenotype than receptor deficiency alone.

$TR\alpha1^{R384C/+}$ mice, with a less potent mutation of TRα, are euthyroid. They display transient growth retardation with delayed ossification. Trabecular bone mass increased with age and adults had osteosclerosis due to a remodeling defect.[149] Remarkably, brief T3 supplementation during growth, at a dose sufficient to overcome transcriptional repression by $TR\alpha1^{R384C}$, ameliorated the adult phenotype.[149] Thus, even transient relief from transcriptional repression mediated by unliganded TRα1 during development has long-term consequences for adult bone structure and mineralization.

$TR\alpha^{AMI}$ mice harbor a floxed *Thra* allele [AF2 mutation inducible (AMI)] and express a dominant-negative mutant $TR\alpha1^{L400R}$ following Cre-mediated recombination. Mice with global expression of $TR\alpha1^{L400R}$ had a similar skeletal phenotype to $TR\alpha1^{PV/+}$ and $TR\alpha1^{R384C/+}$ mice.[154] Furthermore, restricted expression of $TR\alpha1^{L400R}$ in chondrocytes resulted in delayed endochondral ossification, impaired linear growth and a skull base defect.[155] Microarray studies using chondrocyte RNA revealed

changes in expression of known T3-regulated genes, but also identified new target genes associated with cytoskeleton regulation, the primary cilium, and cell adhesion. Restricted expression of TRα1^{L400R} in osteoblasts unexpectedly resulted in no skeletal abnormalities.[155]

In summary, the presence of unliganded or mutant TRα is more detrimental to the skeleton than absence of the receptor. Overall, these studies (1) demonstrate the importance of thyroid hormone signaling for normal skeletal development and adult bone maintenance, and (2) identify a critical role for TRα in bone.

5.4 Targeting TRβ (Fig. 31.4)

Two TRβ$^{-/-}$ strains have been generated with similar skeletal phenotypes.[149,156] Mice lacking TRβ recapitulate RTH with increased T4, T3, and TSH. Juveniles have accelerated endochondral and intramembranous ossification, advanced bone age, increased mineral deposition, and persistent short stature due to premature growth plate closure. Increased T3 target gene expression demonstrated enhanced T3 action in skeletal cells resulting from effects of elevated thyroid hormones mediated by TRα in bone. Accordingly, the phenotype is typical of the skeletal consequences of thyrotoxicosis and adult TRβ$^{-/-}$ mice have progressive osteoporosis.[149,156]

TRβ$^{PV/PV}$ mice with the PV mutation have a severe RTH phenotype with a 15-fold increased T4, 9-fold increased T3, and 400-fold increased TSH.[41,127] Consequently, TRβ$^{PV/PV}$ mice have more severe abnormalities than TRβ$^{-/-}$ mice with accelerated intrauterine growth, advanced ossification, craniosynostosis, and short stature.

Overall, skeletal abnormalities in TRβ mutant mice are consistent with a predominant physiological role for TRα in bone.

5.4.1 *Cellular and Molecular Mechanisms*

The skeletal phenotypes in mutant mice provide compelling evidence of distinct roles for TRα and TRβ in the skeleton and HPT axis. The contrasting phenotypes of TRα and TRβ mutant mice can be explained by the predominant expression of TRα in bone and TRβ in hypothalamus and pituitary. Thus, in TRα mutants, delayed ossification with impaired bone remodeling in juveniles and increased bone mass in adults is a consequence of disrupted T3 action in skeletal T3 target cells, whereas accelerated skeletal development and adult osteoporosis in TRβ mutants result from supraphysiological stimulation of skeletal TRα due to disruption of the HPT axis.[61]

This paradigm is supported by analysis of T3 target gene expression, which demonstrated reduced skeletal T3 action in TRα mutant animals,[127,149,156,157] and increased T3 action in TRβ mutants.[41,149,156,158] The studies further demonstrate that T3 exerts anabolic actions during skeletal development but catabolic actions in adult bone. The data also indicate that effects of disrupted or increased T3 action in bone predominate over skeletal responses to TSH. For example, TRα$^{0/0}$, TRα1$^{PV/+}$, and TRα1$^{R384C/+}$ mice have grossly increased trabecular bone mass even when TSH concentrations are normal, while TRβ$^{-/-}$ and TRβ$^{PV/PV}$ mice are osteoporotic despite markedly increased levels of TSH. Consistent with this conclusion, TRα1$^{-/-}$β$^{-/-}$ double knockout mice, generated in a different genetic background, also display reduced bone mineralization despite grossly elevated TSH.[143] Finally, although TRα is the major TR isoform expressed in bone, TRα$^{0/0}$ mice also remain partially sensitive to T4 treatment, suggesting a compensatory role for TRβ in skeletal cells.[42]

5.4.2 *Downstream Signaling Responses in Skeletal Cells*

GH receptor (GHR) and IGF1 receptor (IGF1R) mRNA was reduced in growth plate chondrocytes in TRα$^{0/0}$ and TRα1$^{PV/+}$ mice and phosphorylation of signal transducer and activator of transcription 5 (STAT5) and protein kinase B (AKT) was also impaired.[127,149] By contrast, GHR and IFG1R expression and downstream signaling were increased in TRβ$^{-/-}$ and TRβ$^{PV/PV}$ mice.[127,156] Studies in primary osteoblasts revealed that T3/TRα1 bound to a response element in intron 1 of the Igf1 gene to stimulate transcription.[159] Thus, GH/IGF1 signaling is a downstream mediator of T3 action in the skeleton in vivo.

Activation of FGFR1 stimulates osteoblast proliferation and differentiation and FGFR3 regulates growth plate chondrocyte maturation and linear growth. *Fgfr3* and *Fgfr1* expression was reduced in growth plates of TRα$^{0/0}$, TRα1$^{R384C/+}$, and TRα1$^{PV/+}$ mice and *Fgfr1* expression was reduced in osteoblasts from TRα$^{0/0}$ and Pax8$^{-/-}$ mice.[30,127,149,156–158] By contrast, *Fgfr3* and *Fgfr1* expression was increased in growth plates of TRβ$^{-/-}$ and TRβ$^{PV/PV}$ mice and *Fgfr1* expression was increased in osteoblasts from TRβ$^{PV/PV}$ mice.[41,149,156,158] Thus, FGF/FGFR signaling is a downstream mediator of T3 action in chondrocytes and osteoblasts.

T3 is essential for cartilage matrix synthesis and heparan sulfate proteoglycans are key components essential for FGF and IHH signaling. Studies in thyroid manipulated rats and TRα$^{0/0}$TRβ$^{-/-}$ and Pax8$^{-/-}$ mice demonstrated reduced HSPG expression in thyrotoxic animals, increased expression in TRα$^{0/0}$TRβ$^{-/-}$ mice and more markedly increased expression in hypothyroid rats and congenitally hypothyroid Pax8$^{-/-}$ mice.[160] Thus, T3 coordinately regulates FGF/FGFR and IHH/PTHrP signaling within the growth plate via regulation of HSPG synthesis.

In neonatal TRβ$^{PV/PV}$ mice, *Runx2* expression was increased in perichondrial cells surrounding the developing growth plate, and in 2-week-old mice *Rankl* expression was decreased in osteoblasts.[161] Although the

findings are consistent with increased canonical Wnt-signaling pathway during postnatal growth, expression of Wnt4 was decreased. Unliganded TRβ physically interacts with and stabilizes β-catenin to increase Wnt signaling but this interaction is disrupted by T3 binding. However, although $TR\beta^{PV}$ similarly interacts with β-catenin its interaction is not disrupted by T3,[162] and this leads to persistent activation of Wnt signaling in $TR\beta^{PV/PV}$ mice despite reduced expression of Wnt4.[161] Tsourdi et al. investigated Wnt signaling in hypothyroid and thyrotoxic adult mice.[163] In hyperthyroid animals bone turnover was increased and the serum concentration of the Wnt inhibitor DKK1 was decreased. In hypothyroid mice bone turnover was reduced and the DKK1 concentration increased. Surprisingly, concentrations of another Wnt inhibitor, sclerostin, were increased in both hyperthyroid and hypothyroid mice.[163] These preliminary studies suggest increased Wnt signaling may lie downstream of T3 action in bone.

6 ROLE OF THYROID HORMONES IN OSTEOARTHRITIS

6.1 Human Population Studies

A GWAS of siblings with generalized OA found a nonsynonymous variant (rs225014; T92A) that identified *DIO2* as a disease-susceptibility locus[164] and an allele sharing approach reinforced evidence of linkage.[165] Replication studies confirmed this by identifying an association between OA and a *DIO2* haplotype containing the minor allele of rs225014 and major allele of rs12885300.[166] The relationship between these *DIO2* SNPs and OA, however, was not replicated in the Rotterdam Study population,[167] and *DIO2* was not identified in recent meta-analyses.[168,169] Nevertheless, additional data suggested the rs225014 risk allele might be expressed at higher levels than the protective allele in OA cartilage from heterozygous patients,[170] possibly because of epigenetic modifications.[171] Increased *DIO2* expression has also been documented by RT-qPCR and immunohistochemistry in cartilage from joints affected by end-stage OA.[170,172] Furthermore, the rs12885300 *DIO2* polymorphism has been suggested to influence the association between hip shape and OA susceptibility.[173] Finally, a metaanalysis studying genes that regulate thyroid hormone metabolism and mediate T3 effects on chondrocytes identified *DIO3* as a disease modifying locus.[166] Together, these data suggest local T3 availability in joint tissues may play a role in articular cartilage renewal and repair, particularly as the associated *DIO2* and *DIO3* polymorphisms do not influence systemic thyroid status.[174] Overall, increased T3 availability may be detrimental to joint maintenance and articular cartilage homeostasis.

6.2 Rodent Models of Osteoarthritis

In studies demonstrating restriction of functional DIO2 enzyme to osteoblasts, we showed *Dio2* mRNA expression does not necessarily correlate with enzyme activity.[34] An important reason for this discrepancy is that DIO2 protein is labile, undergoing rapid degradation following exposure to increasing concentrations of T4 in a local feedback loop.[40] We demonstrated *Dio2* mRNA in growth plate cartilage but could not detect enzyme activity using a high sensitivity assay,[34] whereas others showed *Dio2* mRNA expression in rat articular cartilage,[172] and increased *DIO2* mRNA[172] and protein[170] in human articular cartilage from joints resected for end-stage OA. In each case, however, enzyme activity was not determined. The significance of increased *DIO2* mRNA in end-stage OA cartilage is therefore uncertain; it is also unknown whether increased *DIO2* expression might represent a secondary response to joint destruction or whether it precedes cartilage damage and might be a causative factor in disease progression.

Transgenic rats overexpressing DIO2 in chondrocytes had increased susceptibility to OA following surgical provocation, although enzyme activity was not determined and a causal relationship was not established.[172] This is particularly important because in a previous study, transgenic mice overexpressing *Dio2* in cardiomyocytes displayed only mild thyrotoxic changes,[175] likely because the phenotype was mitigated by T4-induced degradation of the overexpressed enzyme.[40] Furthermore, siRNA-mediated inhibition of *DIO2* in primary human chondrocytes resulted in decreased expression of liver X receptor α (LXRα) and increased expression of interleukin-1β (IL1β) and IL1β-induced cyclooxygenase-2 (COX2) expression.[176] In contrast to previous studies, these findings suggest that suppression of *Dio2* results in a proinflammatory response in cartilage. The increase in *DIO2* expression observed in end-stage human OA[170,172] may, therefore, be a consequence of disease progression resulting from activation of proinflammatory factors, such as NFκB, which stimulates *Dio2*.[177]

Adult $Dio2^{-/-}$ mice have normal articular cartilage and no other features of spontaneous joint damage, but exhibit increased subchondral bone mineral content.[178] In a forced exercise provocation model $Dio2^{-/-}$ mice were protected from articular cartilage damage.[179]

6.3 Thyroid Hormone Action in Articular Chondrocytes

Although chondrocytes resist terminal differentiation in healthy articular cartilage, a process resembling endochondral ossification occurs during OA in which articular

chondrocytes undergo hypertrophic differentiation with accelerated cartilage mineralization.[180] ADAMTS5[181] and MMP13[182] degrade cartilage matrix and these enzymes are regulated by T3 in the growth plate.[183] In articular cartilage, thyroid hormones stimulate terminal chondrocyte differentiation[184] and tissue transglutaminase activity.[185] In cocultures of chondrocytes derived from different articular cartilage zones, interactions between chondrocytes from differing zones were identified that modulated responses to T3 and were mediated in part by PTHrP.[186]

Currently, the pathophysiological importance of these studies is difficult to evaluate. Initial genetic studies suggesting that reduced DIO2 activity is associated with increased susceptibility to OA[164] were based on the assumption that the T92A polymorphism results in reduced enzyme activity.[187] However, studies showing that transgenic rats overexpressing DIO2 in articular cartilage had increased susceptibility to OA[172] were not consistent with this conclusion, and recent findings of allelic imbalance and increased expression of DIO2 protein in OA cartilage[170,171] further suggest increased DIO2 activity may be detrimental for articular cartilage maintenance. On the other hand a large GWAS and recent metaanalyses failed to identify *DIO2* as a disease susceptibility locus for OA,[167–169] while a recent study in primary human chondrocytes suggests an antiinflammatory role for DIO2 in cartilage.[176] Thus, it remains unclear whether variation in *DIO2* plays a role in OA pathogenesis, although the independent identification of *DIO3* as a disease susceptibility locus[166] supports a role for control of local tissue T3 availability in regulation of joint homeostasis.

7 CONCLUSIONS

The skeleton is exquisitely sensitive to thyroid hormones, which have profound effects on bone development, linear growth, and adult bone maintenance.

Thyroid hormone deficiency in children results in cessation of growth and bone maturation, whereas thyrotoxicosis accelerates these processes. In adults, thyrotoxicosis is an established cause of secondary osteoporosis, and an increased risk of fracture has been demonstrated in subclinical hyperthyroidism. Furthermore, even thyroid status at the upper end of the reference range is associated with an increased risk of fracture in postmenopausal women.

An extensive series of studies in genetically modified mice has shown that T3 exerts anabolic actions on the developing skeleton, has catabolic effects in adulthood, and these actions are mediated predominantly by TRα1. The importance of the local regulation of T3 availability in bone has been demonstrated in studies that identified a critical role for DIO2 in osteoblasts to optimize bone mineralization and strength. Translational importance and clinical relevance is highlighted by the characterization of mice harboring deletions or dominant-negative mutations of *Thra*, which accurately predicted the abnormalities seen in patients with RTHα. Moreover, mice with dominant-negative mutations of *Thra* also represent an important disease model in which to investigate novel therapeutic approaches in these patients. In addition to studies in genetically modified mice, analysis of patients with thyroid disease or inherited disorders of T3 action is consistent with a major physiological role for T3 in the regulation of skeletal development and adult bone maintenance. Analysis of $Tshr^{-/-}$ mice and studies of TSH administration in rodents have also suggested that TSH acts as a negative regulator of bone turnover. Nevertheless, the physiological role of TSH in the skeleton remains uncertain, as its proposed actions are not wholly consistent with findings in human disease and mouse models in which the physiological inverse relationship between thyroid hormones and TSH is dissociated.

Recent studies have also identified *DIO2* and *DIO3* as disease susceptibility loci for osteoarthritis, a major degenerative disease of increasing prevalence in the ageing population. These data establish a new field of research and further highlight the fundamental importance of understanding the mechanisms of T3 action in cartilage and bone, and its role in tissue maintenance, response to injury, and pathogenesis of degenerative disease. Precise determination of the cellular and molecular mechanisms of T3 and TSH actions in the skeleton in vivo will require cell-specific conditional gene targeting approaches in individual bone cell lineages. Combined with genome-wide gene expression analysis, these approaches will determine key target genes and downstream signaling pathways and have the potential to identify new therapeutic targets for skeletal disease.

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CHAPTER

32

Disorders and Mechanisms of Ectopic Calcification

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1 INTRODUCTION

Once considered passive precipitation of calcium and phosphate, ectopic calcification is now seen as a complex process actively regulated by several circulating and local factors. These factors maintain vessel and tissue homeostasis, which normally involves trophic inhibition of calcification and is disrupted in pathological disorders. Several rare Mendelian diseases, as well as some common disorders, present with strikingly similar histological findings but vastly different clinical manifestations and pathologic sequelae. Here, we describe the clinical presentations, diagnostics, molecular genetics, and treatment of the known disorders of ectopic calcification, in addition to reviewing experimental models and disease mechanisms.

2 DISORDERS OF ECTOPIC CALCIFICATION

2.1 Generalized Arterial Calcification of Infancy (GACI)

2.1.1 Clinical Presentation and Diagnostic Aspects

Infantile calcification of the arteries was first described by Durante in 1899,[1] and the first report in the English medical literature dates back to 1901.[2] Since then, it has been known by various names, including idiopathic obliterative arteriopathy, infantile calcifying arteriopathy, occlusive infantile arteriopathy, medial coronary sclerosis of infancy, diffuse arterial calcifying elastopathy of infancy, arteriopathia calcificans infantum, and perhaps more commonly as idiopathic infantile arterial calcification (IIAC).[3] Since the identification of the molecular etiology in 2003,[4] the preferred nomenclature has been generalized arterial calcification of infancy (GACI). The incidence of GACI, calculated from the carrier frequency noted in a cohort of over 60,000 exomes from unrelated adults, should be approximately 1 in 200,000 live births, although it is likely that only a fraction of affected infants receive that diagnosis. GACI shows a bimodal age of onset, with about half of all patients presenting in utero or during the first week of life, and the other half presenting later in life, with a median age of onset of 3 months.[5] Common signs of the disease in utero include fetal distress, polyhydramnios, and effusions or hydrops, while a common presentation postnatally includes respiratory distress, cyanosis, and heart failure.[5]

GACI is characterized by diffuse calcification of large- and medium-sized arteries (Fig. 32.1A). Extravascular calcifications can occur around the joints (Fig. 32.1B) in 29% of cases,[6] as well as in the ear lobes,[7–9] myocardium,[10–12] pancreas, liver, and kidneys.[8,13] Skin and retinal findings typical of pseudoxanthoma elasticum (PXE; Section 2.3.1) can also present later in life in individuals who survive infancy.[8,13–16] Elevated inflammatory markers, such as white blood cell count and C-reactive protein, commonly occur, leading to the erroneous diagnosis of sepsis. Despite the best medical care, the mortality rate remains 55% within the first 7 months of life.[6]

GACI is diagnosed through imaging studies, including ultrasonography and echocardiography, that detect vessel echobrightness, as well as computed tomography (CT), which remains the preferred technique to evaluate vascular calcification. In the past, the diagnosis was made by histologic means, either through the biopsy of a medium-sized artery or at autopsy. The characteristic

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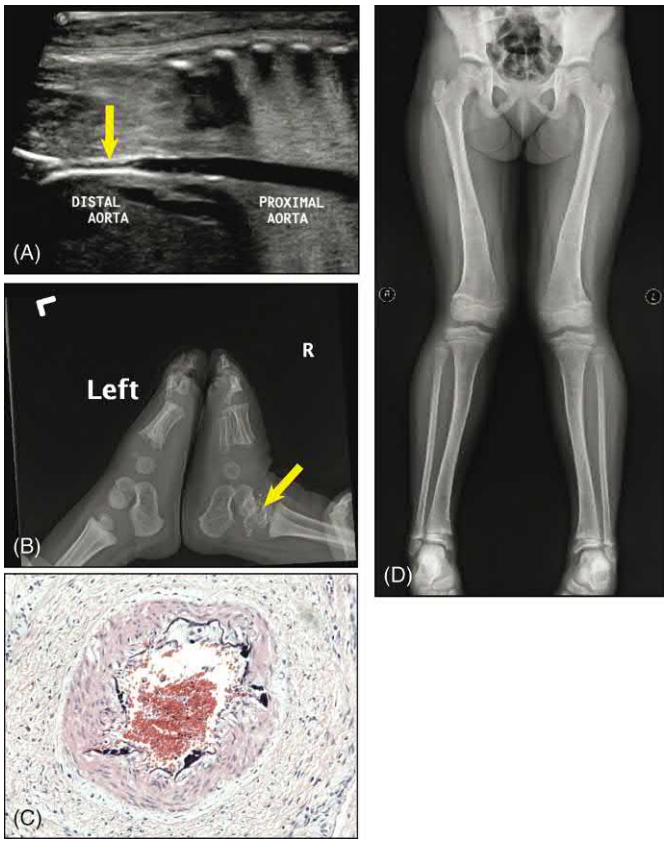

FIGURE 32.1 Common clinical and histological features of generalized arterial calcification of infancy (GACI). (A) Ultrasound imaging showing luminal narrowing and echobrightness *(arrow)* of the distal aorta in a child. (B) Calcification noted in the right ankle joint *(arrow)*. (C) Medium-sized vessel within connective tissue showing calcification of the internal elastic lamina (hematoxylin and eosin stain). (D) Bilateral genu valga in a child with untreated hypophosphatemic rickets due to *ENPP1*-associated GACI.

pathology involves fragmentation and calcification of the internal elastic lamina of large- and medium-sized arteries (Fig. 32.1C); fibrointimal hyperplasia frequently contributes to luminal narrowing and can occur in the absence of calcification. The authors know of one child with biallelic *ENPP1* mutations (see Section 2.1.2) with no arterial calcifications on CT imaging, but with diffuse arterial narrowing, initially diagnosed as fibromuscular dysplasia.

2.1.2 Molecular Genetics

GACI generally results from biallelic mutations in *ENPP1*, which encodes an extracellular ectonucleotide pyrophosphatase that converts ATP into AMP and pyrophosphate (PPi).[4] While mutations in *ENPP1* account for 67% of all cases of GACI, biallelic mutations in *ABCC6* account for 9% of cases,[8] and over 20% of all patients have no known molecular etiology. In general, the different molecular bases of GACI are clinically indistinguishable. However, hypophosphatemic rickets develops in the majority, if not all, of GACI survivors carrying *ENPP1* mutations[6] (Fig. 32.1D), while it is not a complication of GACI due to *ABCC6* mutations (Section 2.3.2). Hearing loss, either conductive, sensorineural, or mixed, can also complicate *ENPP1*–GACI at any point in a patient's lifetime, including the neonatal period.[9] No genotype–phenotype correlation is known for patients with *ENPP1*–GACI, other than all patients homozygous for the p.Pro305Thr died in infancy.[6]

2.1.3 Treatment

Due to the lack of understanding of the disease pathogenesis, treatment options for disorders of ectopic calcification have been limited. One possible therapy consists of bisphosphonates, which have a pyrophosphate backbone that inhibits mineral formation, and a side chain that inhibits mineral resorption. Although the main reason for using bisphosphonates in other conditions, such as osteoporosis, stems from their antiresorbtive effect, the rationale for their use in GACI is related to their antimineralization effect. First-generation bisphosphonates, specifically etidronate, have been proposed as treatment for vascular calcification disorders,[17] and are currently being used to treat GACI. Due to the limited number of GACI patients, the therapeutic efficacy has not been well established, though a retrospective study has shown that etidronate treatment was associated with better clinical outcomes and prolonged survival.[6] The optimal duration of treatment remains to be established, but prolonged bisphosphonate therapy can lead to severe skeletal toxicity.[18] Treatment of subsequent rickets, when performed judiciously, does not lead to worsening of vascular calcification[19]; although overtreatment can result in hypercalciuria and iatrogenic calcification.[13]

2.2 Arterial Calcification due to Deficiency of CD73 (ACDC)

2.2.1 Clinical Presentation and Diagnostic Aspects

In 2011, mutations in *NT5E* were identified in members of three families with symptomatic arterial and joint calcification.[20] Today, a total of 13 patients are followed at the National Institutes of Health, and more patients have been identified around the world.[21–23] Patients typically present in their late teenage years or early adulthood with joint pain, mostly in the hands and feet, but a rheumatology evaluation fails to provide a specific diagnosis despite radiographs showing periarticular calcification.[21,24] Patients later develop claudication, and plain radiographs show massive calcification of the arteries of the lower extremities (Fig. 32.2). Patients lack classical risk factors for cardiovascular disease, such as diabetes or renal insufficiency, but are treated empirically for peripheral vascular disease, either medically or surgically. Isolated patients with arterial calcification due to deficiency of

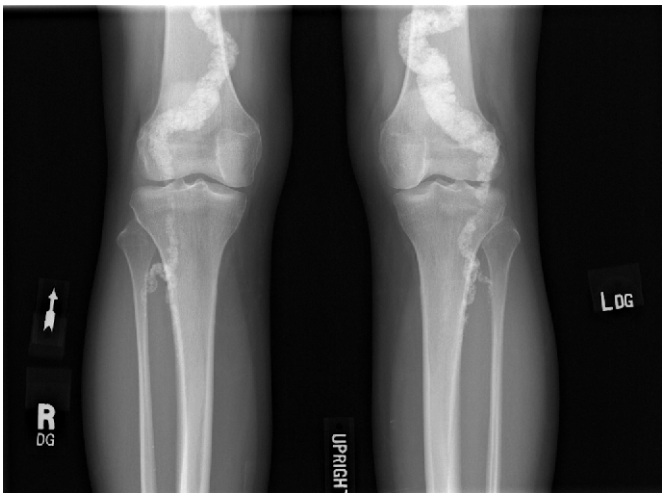

FIGURE 32.2 Calcification of the popliteal and posterior tibial arteries in a patient with arterial calcification due to deficiency of CD73 (ACDC).

CD73 (ACDC) have had calcification of upper extremity vessels,[22] splenic artery, coronary arteries, soft tissue in the neck, and a calcified brain meningioma.

2.2.2 *Molecular Genetics*

ACDC results from biallelic mutations in *NT5E*, which encodes CD73, an ecto-5′-nucleotidase that degrades AMP to adenosine and inorganic phosphate (Pi).[20] Mutations identified in the original three families caused reduced endoplasmic reticulum retention and reduced trafficking of the defective protein to the plasma membrane.[25] No genotype–phenotype correlation is known.

2.2.3 *Treatment*

A phase 1 clinical trial to evaluate the effectiveness of etidronate is currently underway (ClinicalTrials.gov identifier: NCT01585402).

2.3 Pseudoxanthoma Elasticum (PXE)

2.3.1 *Clinical Presentation and Diagnostic Aspects*

PXE is characterized by fragmentation and mineralization of elastic fibers, mainly affecting the skin, retina, and cardiovascular system. In 1881,[26] Rigal first described the skin findings and attributed them to a form of diffuse xanthelasma. Later, Ferdinand-Jean Darier realized that the skin lesions were not xanthomatous, but rather caused by calcification of the elastic fibers; thus, he coined the term PXE.[27] In 1929, the association of the typical skin findings with retinal angioid streaks was independently reported by two Swedish doctors, Ester Grönblad[28] and James Strandberg[29]; the disorder is still sometimes known as Grönblad–Strandberg syndrome.

Although it was initially thought to be an exceedingly rare disease, with older literature mentioning a prevalence of 1 in 1,000,000, a prevalence as high as 1 in 25,000 has been proposed.[30] Based on the allele frequency for the most common *ABCC6* mutation, R1114*, a prevalence as high as 1 in 4450 has been calculated in the Dutch population.[30,31] A founder effect has been reported in the Afrikaner population of South Africa.[32] The female-to-male ratio is approximately 2 to 1.[33]

From a series of 100 patients, the mean age at onset of symptoms was 13.5 years, with mean age at diagnosis of 22.9 years.[33] The first symptoms are almost always cutaneous,[33] in the form of small, asymptomatic, yellow papules that coalesce over time into larger plaques. The appearance of the skin has been described as that of "plucked chicken," "Moroccan leather," "cobblestone," "crépe like," or "pseudoxanthomatous."[34] In more advanced stages, the papules can become obscured by folds of redundant skin.[34] The first site of involvement in 96% of cases is the lateral neck, but the axillae, antecubital fossae, inguinal fossae, and groins can be involved, typically following a cephalad-to-caudal order of progression.[33] Other affected areas include the inner aspect of the lower lip (33% of cases), rectal mucosae, supraumbilical area, flexor surface of the wrist, medial thighs, dorsum of the ankles, penis, and genital labia.[33] In cases with facial involvement, the skin redundancy leads to a "hound dog" appearance.

The typical retinal findings of PXE include angioid streaks, mottled hyperpigmentation (peau d'orange), atypical drusen, and retinal hemorrhages (Fig. 32.3). Angioid streaks are irregular lines that radiate from the optic disc, and are the manifestation of ruptures in Bruch's membrane; they are seen in 83% of cases and start appearing during the second decade of life.[33] Peau d'orange represents a mottled pigmentation of the retinal pigment epithelium best seen in the periphery of the retina; this finding is present in 96% of patients, making it the most common retinal sign of PXE.[33] Other findings include atypical drusen (52% of cases) and hyperpigmented spots on both sides of an angioid streak (owl's eyes).[33] All these findings are asymptomatic until neovascularization occurs, leading to hemorrhages and the consequent visual loss.

Regarding cardiovascular complications, intermittent claudication is present in 30% of cases, angina pectoris in 13%, gastrointestinal bleeding in 8%, mitral valve prolapse in 4%, myocardial infarction in 1%, and cerebrovascular accidents in 1%.[33]

Testicular microlithiasis affects the majority of male patients with PXE,[35] sometimes very early in life.[36] Calcification of the placenta has been described in pregnant women.[37]

The minimal criteria for establishing a clinical diagnosis of PXE requires the presence of angioid streaks in the retina in addition to typical skin findings with characteristic histopathologic changes of mineralization and

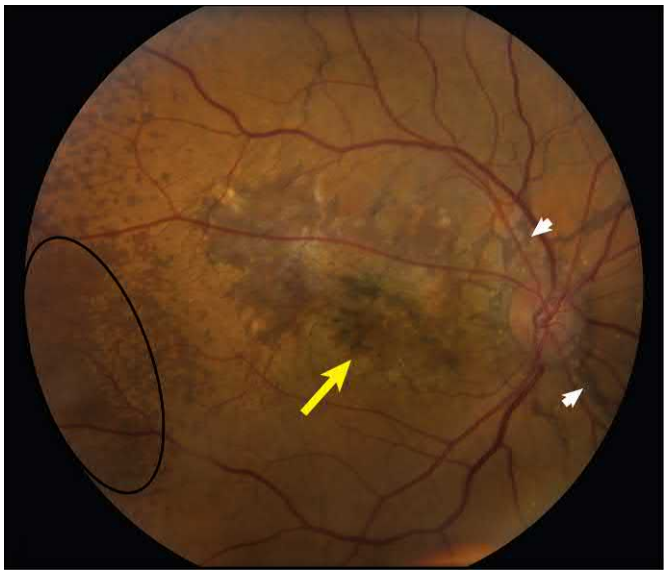

FIGURE 32.3 Fundus photograph of a patient with pseudoxanthoma elasticum (PXE). The *oval* indicates peau d'orange in the peripheral retina, *arrow* indicates macular hemorrhage, and *arrowheads* point to angioid streaks. *Source: Courtesy of Dr. Emily Chew, National Eye Institute, NIH.*

fragmentation of elastic fibers (elastorrhexis).[38] The first finding is that of mineralization of the central core of the elastic fiber, followed by formation of central holes, and then fragmentation of the fibers.[39] Special stains used in light microscopy include Verhoeff–van Gieson (staining elastic fibers black) and calcium stains, such as von Kossa and alizarin red (staining calcium brown black and reddish orange, respectively).

There are several conditions that can cause PXE-like phenotypes. The reported incidence of angioid streaks in patients with sickle cell disease has varied from 1%–2%[40] to 22%,[41] the latter in patients over 40-years old. Angioid streaks have been found in 20% of patients with β-thalassemia[42] (showing a positive correlation with age) and in 10% of patients with sickle thalassemia.[43] Of 40 patients with β-thalassemia, 55% had calcification of the posterior tibial arteries, 20% had skin lesions typical of PXE, and 52% had angioid streaks, with 85% having at least one of the three.[44] The pathology of the skin lesions, based on light microscopy, electron microscopy, and immunohistochemistry is identical to that seen in PXE.[45] Exposure to potassium nitrate (saltpeter) is also associated with skin lesions that are clinically and histopathologically identical to those of PXE.[46,47] Penicillamine use can induce similar skin changes, described as pseudo-PXE[48–50]; there is a case of familial pseudo-PXE in relatives who were taking penicillamine for the treatment of cystinuria.[51] PXE has also been described in three liver transplant recipients,; although liver specimens from donors did not reveal mutations in *ABCC6*.[52] Patients with juvenile Paget disease can also have PXE-like skin lesions[53–56] and retinal findings.[57–59] Finally, an unknown disorder with manifestations of PXE, hyperphosphatemia, hypercalcemia, and nonsuppressed 1,25-dihydroxyvitamin D has been described.[60]

2.3.2 Molecular Genetics

PXE is most commonly caused by mutations in *ABCC6*, but it can also be caused by mutations in *ENPP1*.[8,61] No genotype–phenotype correlation is known,[62] other than patients with *ENPP1*-associated PXE are likely to have experienced rickets during childhood or adolescence. PXE is inherited in an autosomal recessive manner; although historically there have been many reports of autosomal dominant inheritance, cases with unambiguous PXE in two different generations have been proven to represent instances of pseudodominance.[63,64] Polymorphisms in the xylosyltransferase genes have been described as modifiers of PXE phenotype severity.[65] PXE-like disorder with multiple coagulation factor deficiency (OMIM 610842) is an autosomal recessive condition due to mutations in the *GGCX* gene. Similarities to PXE include yellowish papules, retinal angioid streaks and peau d'orange, and dermal elastorrhexis.[66] Differences from PXE include more diffuse skin involvement with cutis laxa over time, no decrease in visual acuity, the presence of coagulation abnormalities, and the finding of mineralization of the periphery—as opposed to the core—of the elastic fiber on electron microscopy.[66]

2.3.3 Treatment

No specific treatment exists. Therapeutic options for choroidal neovascularization include laser photocoagulation, transpupillary thermotherapy, photodynamic therapy, and antiangiogenic agents. Plastic surgery has been performed for cosmetic improvement of skin lesions.[67]

2.4 Familial Chondrocalcinosis Type 2

2.4.1 Clinical Presentation and Diagnostic Aspects

Familial chondrocalcinosis was first described by Zitnan and Sitaj in 1957,[68] during the Ninth International Congress on Rheumatic Diseases in Toronto. It is characterized by early-onset deposition of calcium pyrophosphate dihydrate (CPPD) in cartilage, mainly affecting the knees and wrists (hyaline cartilage), and the menisci, pubic symphysis, and intervertebral disks (fibrocartilage).[69] This crystal deposition is evidenced radiographically, and leads to arthropathy. The clinical patterns described include pseudogout alone (with acute or subacute attacks, 19%), pseudogout with osteoarthritis (chronic osteoarthritis with superimposed attacks, 44%), pseudoosteoarthritis alone (similar to osteoarthritis but with a different pattern of joint involvement affecting the metacarpophalangeal joints, wrists, elbows, and shoulders in a symmetric fashion, 22%),

and pseudorheumatoid arthritis (chronic inflammatory arthritis, 15%).[69] Pseudogout attacks typically start between the late third and early fourth decades.[70] There is radiographic evidence of chondrocalcinosis affecting the knees (93%), pubis symphysis (67%), wrists (59%), and hips (52%).[69] Occasionally, affected individuals can show radiographic evidence of chondrocalcinosis and still remain asymptomatic, while others can have destructive arthropathy necessitating joint replacement.[69] Laboratory studies do not disclose blood abnormalities of mineral metabolism.[69] CPPD crystals can be identified in synovial fluid by their positive birefringence on compensated polarized light microscopy.

Other genetic disorders that have been associated with CPPD deposition include hemochromatosis,[71] disorders causing hypomagnesemia, such as Gitelman syndrome,[72] hypophosphatasia,[73–75] Wilson disease,[76] and alkaptonuria.[77–79]

2.4.2 *Molecular Genetics*

Familial chondrocalcinosis type 2 results from mutations in *ANKH*,[80] which also causes a rare skeletal dysplasia known as craniometaphyseal dysplasia and characterized by hyperostosis, sclerosis of craniofacial bones, and abnormal modeling of the metaphyses of long bones.[81] Both conditions are inherited in an autosomal dominant manner, with chondrocalcinosis likely being caused by gain-of-function mutations, and craniometaphyseal dysplasia by dominant negative mutations.[82] Mutations that lead to craniometaphyseal dysplasia cluster in the C-terminus of *ANKH*, whereas mutations in the N-terminus result in chondrocalcinosis.[83] Craniometaphyseal dysplasia has been reported to cosegregate with chondrocalcinosis in females from an Australian family.[84] An autosomal recessive disorder caused by homozygous mutations in *ANKH* was reported in one family, whose affected members presented with painful periarticular calcification of small joints, progressive spondyloarthropathy leading to ankylosis, osteopenia, mixed hearing loss, intellectual disability, and mild hypophosphatemia.[85]

2.4.3 *Treatment*

There is no specific therapy for the condition. Treatment is symptomatic, including the use of nonsteroidal antiinflammatory drugs and intraarticular or systemic glucocorticoids for the management of pain or inflammation.

2.5 Familial Tumoral Calcinosis (FTC)

2.5.1 *Clinical Presentation and Diagnostic Aspects*

Familial tumoral calcinosis (FTC) is characterized by the development of calcified masses around one or more large joints (Fig. 32.4). It was first described by Giard and Duret in 1898 and 1899, respectively.[86,87] Teutschlaender called the disease lipocalcinogranulomatosis and studied it since the 1930s in Europe,[88] where the condition came to be called Teutschlaender disease. Inclan was the first to report the disease in the American literature, and he coined the term tumoral calcinosis,[89] which subsequently became widely adopted. FTC can be either hyperphosphatemic or normophosphatemic.

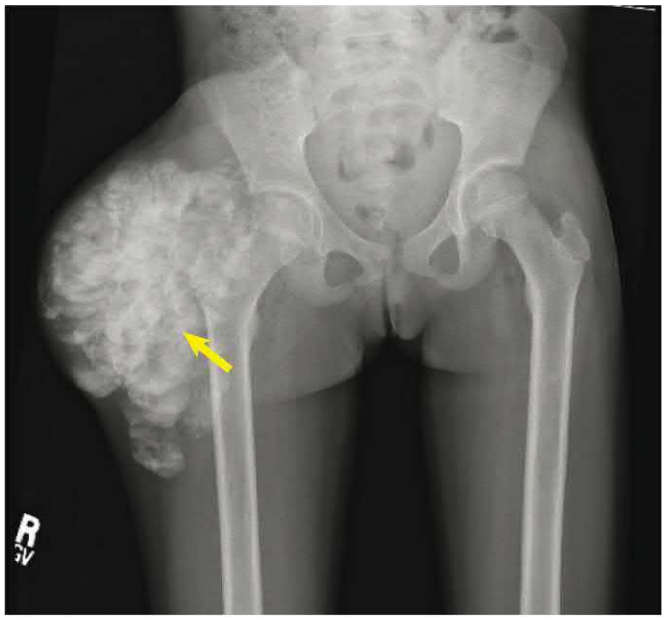

FIGURE 32.4 Patient with familial tumoral calcinosis (FTC) showing an amorphous, multilobulated calcific mass (*arrow*) around the right hip joint. *Source: Courtesy of Drs. Rachel I. Gafni, Michael T. Collins, and Mary S. Ramnitz, National Institute of Dental and Craniofacial Research, NIH.*

A condition known as hyperostosis–hyperphosphatemia syndrome is characterized by cortical hyperostosis, periosteal reaction, and hyperphosphatemia, and is now known to belong to the spectrum of hyperphosphatemic FTC.[90,91] A review of 56 patients with molecularly confirmed hyperphosphatemic FTC found hyperphosphatemic FTC alone in 54% of cases, hyperostosis–hyperphosphatemia alone in 11%, and combined hyperphosphatemic FTC with hyperostosis in 36% of patients.[92] Males and females are equally affected, but hyperostosis is more common in females.[92] Age at presentation was 2–13 years in 78% of cases.[92] Dental involvement was seen in 39% of cases,[92] and included short bulbous teeth with blunted roots, thistle-shaped dental pulps, obliteration of the pulp chamber and root canal, and pulp stones.[93] Vascular calcification was present in 18%, and eye involvement in 16% of patients,[92] including calcification of the eyelids or conjunctiva, band keratopathy, retinal angioid streaks, or optic nerve head drusen.[92,94,95] Testicular microlithiasis has also been described.[96]

The laboratory findings of hyperphosphatemic FTC include increased tubular resorption of phosphate for the degree of hyperphosphatemia, elevated or inappropriately normal 1,25-dihydroxyvitamin D, low intact fibroblast growth factor 23 (FGF23) with markedly increased C-terminal FGF23—indicative of increased FGF23 cleavage—with normal calcium and parathyroid hormone levels. Some patients present with systemic inflammation and increased C-reactive protein.[97]

Normophosphatemic FTC is characterized by the appearance of an erythematous papular eruption during the first year of life (earlier than the typical age of onset of hyperphosphatemic FTC), followed by the appearance of calcified masses in the extremities; patients also show severe conjunctivitis and gingivitis.[98]

2.5.2 Molecular Genetics

Patients and families with hyperphosphatemic FTC have mutations of either the *FGF23*, polypeptide *N*-acetylgalactosaminyltransferase 3 (*GALNT3*), or Klotho (*KL*) genes, all of which encode proteins involved in phosphate regulation. FGF23 is a secreted osteocyte protein, whose posttranslation processing requires GALNT3-mediated mucin-type O-glycosylation. FGF23 acts on the renal tubule via a membrane protein complex comprising the FGF receptor and coreceptor, Klotho, to promote renal phosphate excretion by downregulating type 2 sodium–phosphate cotransporters. FGF23 also decreases intestinal phosphate absorption by inhibiting the renal vitamin D 1α-hydroxylase–mediated synthesis of 1,24-dihydroxyvitamin D (Fig. 32.5). Mutations in *GALNT3* account for 75% of FTC cases, while mutations in *FGF23* and *KL* account for 23 and 2%, respectively. Vascular calcification is more common in patients carrying *FGF23* mutations, while dental involvement is more common in those with *GALNT3* mutations.[92]

Normophosphatemic FTC is caused by biallelic loss-of-function mutations in *SAMD9*, while the newly described *M*yelodysplasia, *I*nfection, *R*estriction of growth, *A*drenal hypoplasia, *G*enital phenotypes, and *E*nteropathy (MIRAGE) syndrome is caused by de novo heterozygous gain-of-function mutations in *SAMD9*.[99]

Regardless of the gene involved, all forms of FTC show autosomal recessive inheritance.

2.5.3 Treatment

Treatment is targeted at decreasing enteral absorption of phosphate or increasing the renal excretion of phosphate. Decreased intestinal absorption can be achieved by dietary phosphate restriction (with a goal of 400–900 mg/day) and use of phosphate binders, such as aluminum hydroxide or sevelamer. Increased renal excretion can be achieved by the use of acetazolamide or probenecid.[93,97,100] The response to treatment is quite variable, with some patients showing complete resolution of lesions, and others showing no improvement. In patients with systemic inflammation, the use of interleukin-1 antagonists can be beneficial.[97] Surgical debulking can be performed in case of pain, deformity, or restriction of joint mobility, but lesions tend to recur.

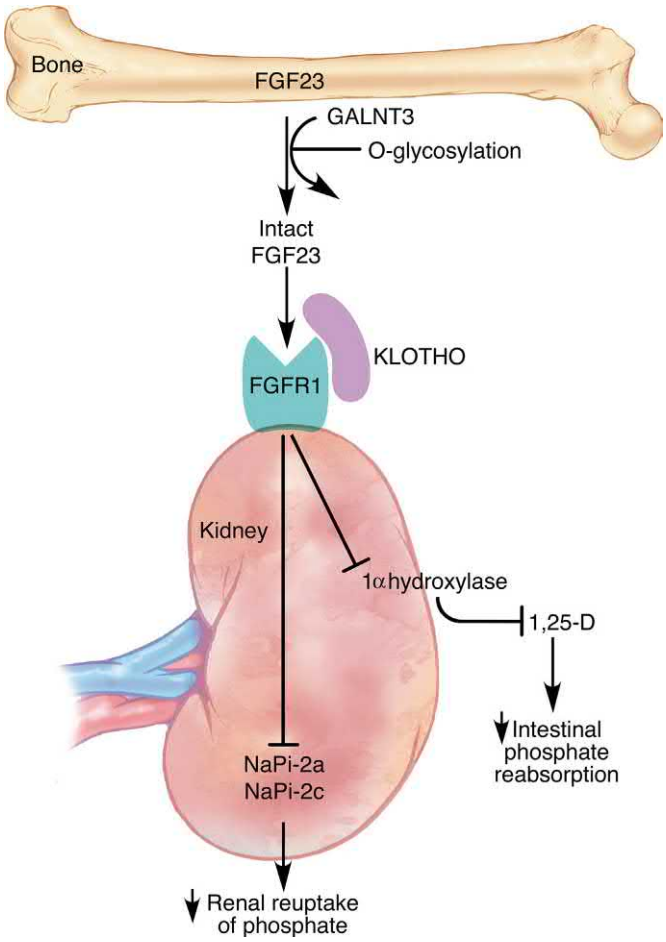

FIGURE 32.5 The roles of *N*-acetylgalactosaminyltransferase (GALNT3), fibroblast growth factor 23 (FGF23), and KLOTHO in phosphate homeostasis. FGF23 undergoes mucin-type O-glycosylation in the Golgi apparatus of osteocytes; this posttranslational modification is needed for the proper secretion of intact FGF23, and protects the latter against cleavage by a furin protease. In the kidneys, FGF23 binds to a receptor/coreceptor complex at the cell surface, and initiates a signaling cascade that downregulates the renal type 2 sodium-phosphate cotransporters and 1α-hydroxylase, thus decreasing renal phosphate reabsorption and intestinal phosphate absorption, respectively.

2.6 Idiopathic Basal Ganglia Calcification (IBGC)

2.6.1 Clinical Presentation and Diagnostic Aspects

Vascular calcification of the basal ganglia was first described by Delacour in 1850,[101] in a 56-year-old man with weakness and spasticity of the lower extremities, as well as tremor.[102] Eighty years later, Karl Theodor Fahr reported the case of an 81-year-old man with a long history

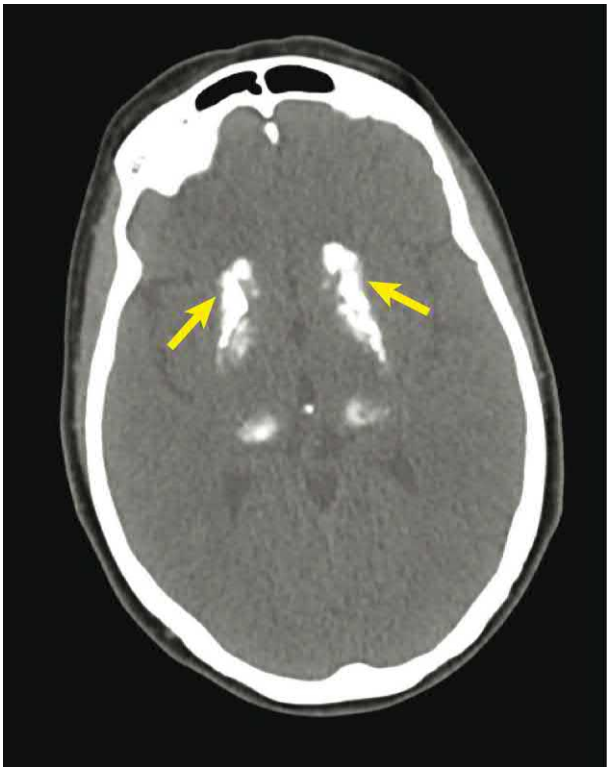

FIGURE 32.6 Brain computed tomography (CT) of a patient with idiopathic basal ganglia calcification (IBGC) showing bilateral calcification *(arrows)* of the basal ganglia.

of dementia, which was a sporadic rather than a familial case. The patient may have had hypoparathyroidism, and the calcification was primarily located in the white matter vasculature, not in the basal ganglia. This, added to the fact that over time the term Fahr disease came to be used for any form of bilateral basal ganglia calcification regardless of etiology, has led to a recommendation against the use of that eponym.[103]

In idiopathic basal ganglia calcification (IBGC), calcifications are found not only in the basal ganglia (Fig. 32.6), but also in the thalamus, dentate nucleus, and centrum semiovale.[104] In a review of 99 cases, 67 were symptomatic at the time of evaluation.[104] The most common symptoms were movement disorders (55%), cognitive defects (39%), speech disorder (36%), cerebellar signs (36%), psychiatric manifestations (31%), pyramidal signs (22%), sensory symptoms (16%), genitourinary symptoms (13%), gastrointestinal symptoms (12%), and seizures (9%). Of the movement disorders, the most common were parkinsonism (57%), followed by chorea (19%), tremor (8%), dystonia (8%), athetosis (5%), and orofacial dyskinesia (3%).[104] The mean age of onset of symptoms is 39 ± 20 years[105]; 25% have onset before the age of 18 years, presenting predominantly with isolated psychiatric or cognitive signs, and 25% have onset after the age of 53 years, presenting mainly with movement disorders.[106] Migraine is found in approximately one in four patients.[106]

Other genetic conditions commonly associated with basal ganglia calcifications include Down syndrome,[107] Cockayne syndrome,[108] mitochondrial disorders,[109] dihydropteridine reductase deficiency,[110] Fried syndrome,[111] Coat's plus syndrome,[112] Raine syndrome,[113] Aicardi–Goutieres syndrome,[114] pseudohypoparathyroidism,[115] Krabbe disease,[116] carbonic anhydrase II deficiency,[117] ISG15 deficiency,[118] CANDLE syndrome,[119] Kenny–Caffey syndrome[120] and Nasu–Hakola syndrome.[121] Lipoid proteinosis is typically associated with calcification of the amygdalae, but sometimes the calcium deposits can extend to the basal ganglia.[122]

2.6.2 Molecular Genetics

There are currently four genes that are known to account for the IBGC phenotype: *SLC20A2*, *PDGFB*, *PDGFRB*, and *XPR1*, all exhibiting autosomal dominant inheritance. Approximately 41%–50% of familial cases were initially reported to be caused by heterozygous mutations in *SLC20A2*,[123,124] with 4.3% of sporadic cases caused by mutations in that gene.[123] However, a recent French series described *SLC20A2* mutations in 20% of familial IBGC and 17.2% of sporadic cases, *PDGFB* mutations in 10% of familial and 13.8% of sporadic cases, and *PDGFRB* mutations in 5% of familial and 6.9% of sporadic cases.[125] Mutations in *XPR1* account for less than 8% of patients who are negative for those three genes.[126] Calcification tends to be more severe in patients carrying mutations in *SLC20A2* than in those with *PDGFRB* mutations.[105,106]

2.6.3 Treatment

No specific treatment exists for IBGC. Treatment is targeted to the symptoms, including medications for movement disorders, psychiatric symptoms, seizures, and migraines. Etidronate use was attempted in one patient, leading to improved speech and gait, but there was no improvement in spasticity, dystonia, or ataxia, and no reduction in intracranial calcification.[127] Alendronate was recently used in seven patients, with good tolerance and either stability or subjective improvement of symptoms, particularly in younger patients.[128] The benefits of bisphosphonates in this condition, if any, are still equivocal.

2.7 Keutel Syndrome

2.7.1 Clinical Presentation and Diagnostic Aspects

Keutel et al. first described this condition in 1972 in a brother and sister born from a consanguineous union.[129] The disease is characterized by diffuse cartilage calcification, including the nose, pinna, larynx, trachea, bronchi, and costochondral junctions,[129] which can lead to persistent respiratory symptoms (68%), recurrent sinusitis and otitis media (67%), and tracheobronchial stenosis (50%).[130]

Patients also have stippled epiphyses of long bones, peripheral pulmonary stenosis (72%), cardiac murmur (69%), and hearing loss (91%),[130] the latter typically mixed. Midface hypoplasia with a flat nasal bridge confers a classic facial appearance known as Binder phenotype.[131] Brachytelephalangy, with shortening and broadening of the first through fourth distal phalanges and sparing of the fifth finger, is considered highly specific for Keutel syndrome, and is present in 75% of patients.[132] Autopsy of the original brother described by Keutel revealed calcification of the internal elastic lamina of the pulmonary, coronary, hepatic, renal, meningeal, and cerebral arteries.[133] Long-term follow-up of patients into adulthood identified multiple erythematous macular skin lesions located in the trunk, neck, dorsum of hands, and elbows, typically appearing after the age of 30 years .[134] Adult patients can also develop massive bullous emphysema, severe systemic hypertension, and short-term amnesia.[134]

The main differential diagnosis is that of brachytelephalangic chondrodysplasia punctata (CDPX1) due to arylsulfatase E deficiency, as this condition can also be accompanied by the Binder phenotype, epiphyseal stippling, tracheal calcification, and brachytelephalangy[135]; although the latter tends to affect all digits,[132] sparing of the fifth distal phalanx has also been described in CDPX1.[135] Another close differential diagnosis is pseudowarfarin embryopathy due to vitamin K epoxide reductase deficiency, as patients with this syndrome also have digital hypoplasia, nasal hypoplasia, and stippled epiphyses; those patients, however, also have coagulation abnormalities.[136] Other genetic conditions associated with auricular calcification include Primrose syndrome[137] and juvenile Paget disease.[138]

2.7.2 Molecular Genetics

Keutel syndrome is an autosomal recessive condition caused by mutations in matrix Gla protein, encoded by *MGP*.[139] The glutamate residues of matrix Gla protein are converted to γ-carboxyglutamate by the action of γ-glutamyl carboxylase (GGCX), with vitamin K as a cofactor for the reaction. Decreased levels of carboxylated matrix Gla protein have been reported in a patient.[140] No genotype–phenotype correlation is known to date.

2.7.3 Treatment

No specific treatment is available. In particular, vitamin K supplements failed to increase circulating levels of carboxylated matrix Gla protein.[140]

2.8 Singleton–Merten Syndrome (SMS)

2.8.1 Clinical Presentation and Diagnostic Aspects

In 1973, Edward Singleton and David Merten first described the condition that now carries their name. This disorder is characterized by aortic and valvular calcification (10/11, 91%; Fig. 32.7A), cardiac arrhythmia (6/11, 55%; Fig. 32.7A), subungual calcification (3/8, 38%), acroosteolysis (6/9, 67%; Fig. 32.7B), short stature (6/9, 67%), osteopenia (9/10, 90%, Fig. 32.7A), scoliosis (3/10, 30%), wide medullary cavities of the phalanges (9/10, 90%), spontaneous tendon rupture (6/11, 55%), joint subluxation (8/9, 89%), glaucoma (5/10, 50%), psoriasiform rash (8/9, 89%), and early loss of secondary dentition (10/11, 91%).[141]

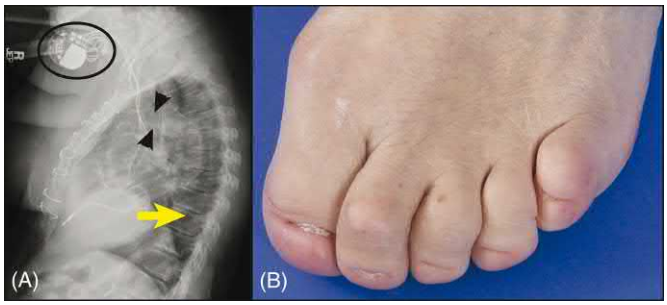

FIGURE 32.7 Typical clinical manifestations associated with Singleton–Merten syndrome (SMS). (A) A pacemaker generator is noted *(oval)*, implanted due to the presence of bradyarrhythmia. The aorta is markedly calcified (porcelain aorta, *arrowheads*). The vertebral bodies show evidence of low–bone mineral density *(arrow)*. In addition, sternotomy wires can be seen, reflecting prior open heart surgery for mitral valve replacement. (B) Hypoplastic (first toe) and aplastic (second to fifth toes) nails.

2.8.2 Molecular Genetics

Currently, only heterozygous mutations in *IFIH1*[142] or *DDX58*[143] account for this autosomal dominant condition. Mutations in either gene lead to interferon signature upregulation. Both genes encode components of the RIG-I-like receptor (RLR) pathway involved in antiviral innate immunity. A third component of this pathway is encoded by *DHX58*, not yet associated with a human disease. The authors have evaluated a family with Singleton–Merten syndrome (SMS) without mutations in the two known genes, so mutations in other genes, particularly those participating in the RLR pathway, could lead to SMS. Regarding genotype–phenotype correlation, dental involvement has not been described in those carrying *DDX58* mutations.[143]

2.8.3 Treatment

No specific treatment is known, but medications that modulate interferon pathway activation might be beneficial.

2.9 Mönckeberg Medial Calcific Sclerosis

Mönckeberg medial calcific sclerosis is characterized by calcific deposits in the small- and medium-sized muscular arteries that occurs independently of inflammation or atherosclerosis. As the intima is not involved, luminal

narrowing does not occur. It was originally described by Johann Georg Mönckeberg in 1903. This type of calcification is typically associated with chronic kidney disease, diabetes, aging, osteoporosis, and vitamin D toxicity. Although historically considered an incidental finding without clinical repercussions, it has more recently been described as a risk factor for cardiovascular disease. Upon histopathological review of cases of Mönckeberg sclerosis, all had calcification of the internal elastic lamina, while calcification of the media was frequent but not universal. Thus, among the different causes of acquired vascular calcification, Mönckeberg sclerosis most closely mimics the pattern of involvement seen in hereditary causes of vascular calcification.

3 ANIMAL MODELS

Animal models of ectopic calcification can produce critical insights into disease mechanism, uncover novel therapeutic targets, and provide evidence for preclinical drug efficacy. Here, we describe the rodent models that have been generated to recapitulate human diseases of pathological ectopic calcification.

3.1 GACI, PXE, and ACDC

The first sign of calcification in GACI, PXE, and ACDC mouse models occurs in the fibrous capsule surrounding their vibrissae (whiskers) on their snouts. While there is no human equivalent for this fibrous structure, it has been well established as an early biomarker for ectopic calcification. Mice harboring biallelic loss-of-function mutations in *Enpp1* manifest robust vibrissae capsule calcification by the age of 1 month and variable aortic and kidney calcification by the age of 5 months. *Enpp1*-mutant mice also develop debilitating extraarticular joint capsule calcification and ankylosis in their forelimbs and vertebral column, leading to progressively limited mobility and death, typically within 6–8 months. The architecture and mineralization of the long bones are disrupted in *Enpp1*-mutant mice, emphasizing the importance of ENPP1 in both bone and vessel homeostasis. Specifically, *Enpp1*-mutant mice have reduced trabecular mass and cortical bone thickness with hypomineralization in the femur and tibia. The bone phenotype in *Enpp1*-mutant mice has been attributed to increased plasma FGF23 levels, and consequently decreased circulating calcium and phosphate,[144] though the mechanism by which *Enpp1* mutations lead to excessive FGF23 production remains unknown.

Like *Enpp1*-mutant mice, *Abcc6*-knockout mice develop ectopic fibrous capsule vibrissae calcification, but with a milder and more delayed presentation. When aged beyond 1 year, *Abcc6*-knockout mice develop focal and sporadic calcification in the skin, eyes, vessels, and other solid organs, recapitulating the common adult-onset and often indolent course of human PXE.[145] *Nt5e*-knockout mice develop minor vibrissae capsule calcification at the age of 1 year approximately without evidence of other ectopic calcification.[146]

Though the phenotype of fibrous capsule vibrissae calcification has variable onset and severity, the observation that it is shared among *Enpp1*-, *Abcc6*-, and *Nt5e*-mutant mice indicates the potential for a unifying disease mechanism. Nevertheless, genotypic heterogeneity cannot be excluded. While it is already established that ENPP1 and CD73 work in the same metabolic pathway, ENPP1 breaking down ATP to AMP, the substrate for CD73, the role of ABCC6 remains unknown. These data suggest that ABCC6 is also integral to the extracellular ATP metabolism pathway.

Use of an acceleration diet composed of high phosphate, low magnesium or warfarin, and vitamin K1 stimulates the *Enpp1*- and *Abcc6*-mutant mouse models to develop multiorgan pathological calcification within 1–6 months.[147, 148] Whereas wild-type mice do not typically calcify when placed on an acceleration diet, mice harboring biallelic *Enpp1* or *Abcc6* mutations exhibit robust aberrant vascular and solid organ calcification.[147-149] *Nt5e*-knockout mice develop extraarticular joint capsule calcification at the age of 1 year approximately when provoked with an acceleration diet.[150]

As diet modification exacerbates disease presentation in the mice, manipulation of the dietary mineral content has also been attempted to attenuate disease progression, specifically in *Abcc6*-knockout mice. While variation in dietary calcium or phosphate does not affect calcification in this mouse model, increasing magnesium reduces vessel calcification in the heart and kidney at the age of 1 year[151] and prevents new, but does not reverse existing, calcium phosphate deposition.[152,153] The mechanism by which high magnesium modulates the calcification process in vivo remains unclear, although it has been shown that magnesium can physically disrupt the crystal lattice, slowing hydroxyapatite formation.[154] Importantly, magnesium and other serum minerals, including phosphate, sodium, calcium, and chloride, are normal in *Abcc6*-mutant mice,[155] indicating that magnesium is modifying disease presentation likely through a parallel pathway instead of correcting a basic defect in the disease.

In addition to high-dose magnesium, nonhydrolyzable analogs of inorganic pyrophosphate, such as bisphosphonates, are also known to directly disrupt calcium and phosphate precipitation and deposition.[156] First-generation bisphosphonates, specifically etidronate, have been proposed as treatments for vascular calcification disorders.[17] A recent study reported that treating *Enpp1*-mutant mice with etidronate (100 μg/kg intraperitoneally) twice a week did not resolve the calcification phenotype.[157] However, high-dose oral etidronate (240 mg/kg/day) was effective in attenuating

fibrous capsule vibrissae calcification in *Abcc6*-mutant mice.[158] The etidronate dose used in the latter mouse trial was 12 times higher than the corresponding dose used to treat osteoporosis in humans, and it resulted in significant changes to bone microarchitecture.[158]

While treatment strategies have focused on disrupting calcium phosphate precipitation, correction of the basic defect has also been pursued. An enzyme replacement strategy with recombinant ENPP1–Fc fusion protein restored circulating serum pyrophosphate levels and prevented mortality and vascular calcification in the aorta, heart, and coronary arteries in *Enpp1*-mutant mice fed an acceleration diet.[159] When the ENPP1–Fc treatment was discontinued, pathologic vascular calcification slowly reemerged but did not lead to death, suggesting that early intervention during a critical interval can extend survival. The efficacy of ENPP1–Fc enzyme replacement in other disorders with defects in extracellular ATP metabolism or more common disorders with established deficiencies in inorganic pyrophosphate remains to be determined.

Overexpressing *Alpl*, the gene that encodes tissue nonspecific alkaline phosphatase (TNAP), in vascular smooth muscle cells has also been used as a model to recapitulate the GACI phenotype and provide insight into a novel therapeutic target. Among other things, TNAP degrades pyrophosphate, the main negative regulator of calcification, and is increased in cell lines derived from GACI, PXE, and ACDC patients.[20,45] TNAP-overexpressing mice have extensive aortic calcification, high blood pressure, cardiac hypertrophy, and increased mortality.[160] This mouse model has been treated with the pharmacological small-molecule TNAP inhibitor SBI-425 to suppress the genetically induced, high TNAP levels and successfully prevent the pathologic calcification.[160] Genetic manipulation to knockout TNAP in *Enpp1*-mutant mice also ameliorates the calcification phenotype, specifically the intervertebral mineral deposits,[161] further establishing TNAP as a druggable target in an endogenous disease model. Use of TNAP inhibitors in other mouse models in which elevated TNAP has been noted in patients' cells are currently underway.

3.2 Chondrocalcinosis and Craniometaphyseal Dysplasia

As in humans, mice with mutations in *Ank*, which encodes a transmembrane pyrophosphate transporter, manifest variable phenotypes with the unifying trait of ectopic calcification. Mice with complete *Ank* deficiency develop extraarticular joint capsule calcification and progressive ankylosis of the vertebral column, leading to progressive joint immobility and complete rigidity and death by the age of 6 months.[162-164] Mice with joint-specific knockout of *Ank* have a delayed onset, but eventually develop the same characteristic phenotype as the complete *Ank*-knockout mice, demonstrating that, although *Ank* is expressed in many tissues, local *Ank* deficiency is sufficient to mimic the disease state.[164]

While the ectopic calcification in *Ank*-deficient mice is composed of hydroxyapatite crystals, the crystals found in humans with chondrocalcinosis consist of CPPD. This fundamental difference in pathology is a consequence of the underlying genetic mutations; while *Ank* deficiency in mice results in loss of function of the transporter and decreased extracellular pyrophosphate, human chondrocalcinosis mutations are likely gain of function and lead to an accumulation of extracellular pyrophosphate.[164] These conclusions are supported by data showing that human disease–causing chondrocalcinosis mutations retain normal transport activity of pyrophosphate in *Xenopus* oocytes and can partially rescue the joint calcification phenotype in *Ank*-knockout mice.[82]

In recent studies, closer examination revealed that *Ank* complete knockout mice exhibit some characteristic features of craniometaphyseal dysplasia patients, including increased skull bone thickness, foramen magnum narrowing, middle ear bone fusion, and decreased trabeculation of femur metaphyses, but do not fully recapitulate other main features of the human disease.[82] A new mouse model with a homozygous in-frame deletion in exon 9 of *Ank* more thoroughly resembles craniometaphyseal dysplasia; these mutant mice develop craniofacial and mandibular hyperostosis, obliteration of nasal sinuses, and flared metaphyses.[165] Of note, craniometaphyseal dysplasia patients have heterozygous, not homozygous, mutations in *ANKH*.[81] Mice harboring this knockin mutation in the heterozygous state developed a mild skeletal phenotype by the age of 1 year, but no other significant phenotypic differences. Taken together, these findings suggest that *ANKH* mutations causing craniometaphyseal dysplasia are not merely loss of function and might in fact be dominant negative.[82] Indeed, there is evidence to suggest that such mutations cause defects in osteoclastogenesis and bone resorption in addition to dysregulated pyrophosphate transport.[166]

Daily injections of phosphocitrate, a structural pyrophosphate analog that potently inhibits hydroxyapatite formation,[167] block ectopic calcification and mitigate joint immobility in *Ank* complete knockout mice.[168] Phosphocitrate prevented new calcification, but did not reverse already established calcification; importantly, there were no adverse effects on bone mineralization.[168]

Ank-knockout mice have a phenotype remarkably similar to that of *Enpp1*-knockout mice, although *Enpp1*-mutant mice have a more extensive hypermineralization phenotype, specifically in the phalanges, and develop pathology in other soft tissue areas, such as the Achilles tendon.[169] *Enpp1*- and *Ank*-deficient mice also have identical expansion of the acellular cementum,[170] the mineralized tissue that surrounds the tooth and anchors it to the

periodontal ligament. As acellular cementum is highly sensitive to local pyrophosphate levels, these findings suggest that both ENPP1 and ANK act in resident cells to maintain tissue homeostasis.[170,171]

Mice deficient for both *Ank* and *Enpp1* qualitatively show more extensive vertebral column calcification than the single mutants alone,[169] suggesting that ANK and ENPP1 have independent effects on extracellular PPi concentrations. There is evidence that ENPP1 can function both extracellularly and intracellularly,[172-174] indicating that intracellular ENPP1 might be generating pyrophosphate for export by ANK. Further studies with double *Ank*- and *Enpp1*-deficient mice are currently underway to probe if ENPP1 and ANK work in series, in parallel, or both (personal communication with Drs. Brian L. Foster and Emily Chu, College of Dentistry, The Ohio State University; and National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH, respectively).

Interestingly, mice with inactivating mutations in *Ent1*, which encodes a facilitative diffusion carrier responsible for the movement of hydrophilic nucleosides, such as adenosine across the plasma membrane, also develop ectopic mineralization of the paraspinal ligament that extends into the intervertebral discs.[175] *Ent1*-mutant mice develop a strikingly similar phenotype to *Enpp1*- and *Ank*-deficient mice, though 6 months delayed.[175] *Ent1*-mutant mice have increased circulating adenosine and pyrophosphate levels and decreased *Alpl*, *Enpp1*, and *Ank* expression in the local tissue that calcifies. Adenosine decreases the expression of *Alpl*,[20] the gene encoding TNAP, and this could explain the increased circulating pyrophosphate; however, it is unclear if the decreased expression of *Enpp1* and *Ank* is primary or compensatory. In any event, these overlapping mouse phenotypes illustrate the complex and poorly delineated intersection of pyrophosphate production and intracellular adenosine signaling.

3.3 Tumoral Calcinosis

Tumoral calcinosis is caused by biallelic inactivating mutations in *Galnt3*, *Fgf23*, or *Klotho*; mouse models have been generated to disrupt these genes to define their functions and probe the interaction of phosphate-regulating factors in vivo. Mutant mouse models develop hyperphosphatemia secondary to increased renal tubular phosphate reabsorption with associated ectopic subcutaneous soft tissue and vascular calcification, in addition to increased or inappropriately normal 1,25-dihydroxyvitamin D levels.[176-178] *Galnt3*- and *Fgf23*-mutant mice have decreased serum concentrations of intact (functional) Fgf23, as their basic defect is in Fgf23 processing, whereas *Klotho*-deficient mice have elevated Fgf23 levels, reflecting compensation for defective signaling at the FGF receptor.[178,179]

By the age of 12 weeks, the *N*-ethyl-*N*-nitrosourea (ENU)–mutated *Galnt3* mouse presents with widespread calcification in the cutaneous striated muscle, heart, kidney, tongue submucosa, and in the vasculature localized to the aorta and testis, and closely recapitulates the clinical features of tumoral calcinosis.[177] The *Galnt3*-knockout mouse model, however, only develops ectopic calcification when provoked with a high-phosphate diet.[180] Nicotinamide, which is hypothesized to lower serum phosphate by decreasing activity of sodium-dependent phosphate reuptake cotransporters in the gut[181] and kidney,[182] was used to treat *Galnt3*-knockout mice initially fed a high-phosphate diet. Although the nicotinamide modestly prevented new calcification, it did not reverse existing calcium deposits and in fact increased calcium content in the heart. It was shown that nicotinamide further decreased circulating levels of intact Fgf23, likely as a compensatory mechanism to increase phosphate reabsorption in the kidney in response to the drug.[183] These findings emphasize the tight regulation of this pathway.

In addition to the common features of tumoral calcinosis, mice deficient in *Fgf23* or *Klotho* also present with extensive occlusive aortic calcifications, vascular and parenchymal renal calcification, thickening and calcification of the auditory ossicles, pulmonary calcification with emphysematous changes, skin atrophy, osteopenia, hypercalcemia, hypoglycemia, and early death.[176,178,184,185] The additional phenotypic features and premature death of *Fgf23*- and *Klotho*-deficient mice represent a more severe phenotype than that of patients with tumoral calcinosis, and suggest that rodent models, compared with humans, are more sensitive to disruptions in this pathway and less likely to compensate for deficiencies in phosphate-regulating factors.

However, these mouse models still provide valuable insights into disease mechanism and potential therapeutic interventions. *Fgf23*-knockout mice fed a low-phosphate diet had decreased serum phosphate and 50% of mutants survived longer; although they still demonstrated growth retardation, hypoglycemia, and elevated 1,25-dihydroxyvitamin D levels; ectopic calcifications were not evaluated in this study.[176] In a trial with more severe dietary restrictions of phosphate, phosphate deficiency corrected the hyperphosphatemia, prevented vascular calcification, and rescued the lethality in *Fgf23*-knockout mice.[186]

Modulating vitamin D activity and levels has also been attempted as therapy for tumoral calcinosis mouse models. Genetically ablating vitamin D activity by knocking out the gene encoding an activator of vitamin D synthesis or 1α-hydroxylase, or inactivating the vitamin D receptor in *Fgf23*-knockout mice eliminates the soft tissue and vascular calcification and ameliorates other aspects of the phenotype, including body weight, skin atrophy, and premature death.[187-189] These genetic models corrected

both the hyperphosphatemia and high–vitamin D levels in the *Fgf23*-mutant mice and therefore did not assess the independent contributions of excessive serum phosphate and vitamin D on vascular calcification. Restricting only vitamin D in mouse models of tumoral calcinosis has had variable effects. Lowering vitamin D by dietary restriction failed to correct the hyperphosphatemia or the vascular calcification, although it did extend survival, in *Fgf23*-null mice,[186] it also prevented ectopic kidney calcification and restored normal body weight in *Klotho*-mutant mice.[190] Taken together, these data implicate both phosphate and vitamin D as predominant mediators of the tumoral calcinosis phenotype. Although the therapeutic efficacy of lowering vitamin D levels remains unclear, these studies imply that decreasing phosphate reduces ectopic calcification.

In other studies, the mineralocorticoid receptor antagonist spironolactone reduced vascular and soft tissue calcification and slightly increased the life span of *Klotho*-deficient mice, without significantly affecting circulating phosphate, vitamin D, FGF23, or calcium concentrations.[191] It has been suggested that spironolactone acts locally to decrease expression of genes involved in procalcific reprogramming and differentiation in the vessel wall.[191] These data indicate that ectopic calcification is not controlled only by circulating phosphate and calcium, but that cellular intrinsic factors also mediate pathologic calcification. Recently, independent supplementation of sodium chloride, ammonium chloride, or bicarbonate also mildly attenuated tissue calcification and extended the life span of *Klotho*-deficient mice,[192-194] suggesting that dysregulation of extracellular volume and pH can influence the disease phenotype.

There is no mouse model for normophosphatemic FTC, as the *SAMD9* ortholog was lost in mice due to a genomic rearrengement.[195]

3.4 Idiopathic Basal Ganglia Calcification

Four genes, *SLC20A2* (*PIT2*), *XPR1*, *PDGFRB*, and *PDGFB*, have been implicated in IBGC. Homozygous *Slc20a2*-knockout mice present with brain calcification localized to arterioles, specifically in the thalamus, basal ganglia, and cortex, by the age of 19 weeks.[196] *Slc20a2*-knockout mice also developed microphthalmia, calcified cataracts and optic nerve tissue, and moderate-to-severe hydrocephalus, phenotypes not typically seen in IBGC patients.[197] *Slc20a2* mice that are heterozygous for the knockout allele more closely recapitulate the human phenotype and develop brain vessel calcification later and without the additional manifestations of homozygous *Slc20a2*-knockout mice.[197]

While serum phosphate and calcium concentrations did not differ among wild-type, heterozygous, and homozygous *Slc20a2* mice, cerebrospinal fluid phosphate levels were trending high and were significantly increased in heterozygous and homozygous *Slc20a2*-mutant mice, respectively.[197] Decreased phosphate import was also observed in vascular smooth muscle cells with experimentally knocked down *Slc20a2*, suggesting that the calcification phenotype is caused by a combination of high circulating phosphate in the cerebrospinal fluid and defects in the local cell population.[197] *Slc20a2*-mutant mice have not been subjected to behavioral or neuropsychiatric testing to see if calcification in brain arterioles leads to functional consequences. *Xpr1*-mutant mice are commercially available but have not yet been evaluated for pathologic calcification or a corresponding phenotype.

While mutations in *Slc20a2* suggest that IBGC is caused by a combination of circulating and local disruptions in phosphate homeostasis, mice completely null for *Pdgfrb* and *Pdgfb* exhibit an inability of pericyte recruitment to developing microvessels; this leads to vascular dysfunction and perinatal death, and implicates a role for the blood–brain barrier maintenance in disease pathogenesis.[198] Central and peripheral nervous system–specific *Pdgfrb*-knockout mice have reduced social behavior and increased locomotor activity, reminiscent of some atypical neurological findings seen in IBGC patients; however, brain calcification was not reported.[199] A mouse model with a hypomorphic allele of *Pdgfb* presented with calcific foci in the midbrain and thalamus at 4 months that expanded with age. Although the blood–brain barrier integrity was not directly tested, these hypomorphic *Pdgfb* mice were rescued by overexpression of PDGFB in the endothelium.[200] Supporting the role of blood–brain barrier defects in disease pathogenesis, fibrinogen depositions at autopsy have been associated with areas of calcification of one IBGC patient, indicating increased permeability of the blood–brain barrier. However, it is uncertain if this blood–brain barrier deficit is a cause or a consequence of the underlying pathology.[201]

Also to note, knocking out *Occludin*, an integral tight junction component of the blood–brain barrier, in mice results in cerebellar and basal ganglia calcification, similar to that seen in the mouse models of IBGC.[202] Taken together, these findings strongly implicate phosphate homeostasis[203] and the PDGFB–PDGFRB signaling pathway[204] in ectopic brain calcification. Though the intersection of these pathways remains unclear, it has been reported that PDGFB increases the expression of *Slc20a2* in culture.[205]

3.5 Keutel Syndrome

Homozygous ablation of *Mgp*, the gene encoding the vitamin K–dependent γ-carboxylated matrix Gla protein, results in extensive arterial and cartilaginous calcification in mice, recapitulating the Keutel syndrome phenotype.[206] Calcification develops at the age of 2–3 weeks approximately and is limited to elastic and muscular

arteries and cartilaginous structures; the phenotype rapidly progresses and results in aortic rupture and death within 2 months.[206] There is evidence of both elastic fiber calcification and cartilaginous metaplasia that can lead to ossification of affected vessels.[206]

In addition to genetic ablation of the gene, matrix Gla protein has also been experimentally decreased by administration of exogenous warfarin, a vitamin K reductase inhibitor.[207] Rats treated with warfarin and concurrent vitamin K1 had decreased extrahepatic vitamin K but normal vitamin K–dependent blood clotting factors and coagulation.[207,208] Warfarin treatment resulted in extensive arterial calcification that did not regress when warfarin was removed,[207] but that did regress when the rats were repleted with vitamin K.[209] Mice with deficiency of osteoblast-specific *Ggcx*, the gene necessary for GGCX that, along with vitamin K, modifies and activates matrix Gla protein, also presented with aberrant calcification.[210] Indeed, phenotypic similarities between the effects of genetic and environmental mediators of matrix Gla protein illustrate the importance of this factor in suppressing pathologic calcification.

3.6 Singleton–Merten Syndrome

An ENU-mutated mouse model with a heterozygous gain-of-function mutation in *Ifih1*, which encodes the intracellular viral sensor MDA5 that has recently been associated with SMS, predominantly develops lupus-like nephritis and systemic autoimmune symptoms without a viral insult.[211] Although these mice do not closely recapitulate the SMS, *Ifih1*-mutant mice develop spontaneous multiorgan inflammation and liver calcification.[211] These mice have not been evaluated for a more extensive calcification phenotype.

3.7 Chronic Kidney Disease, Diabetes, and Aging

Common disorders of vascular calcification, including chronic kidney disease, diabetes, and aging, have also been studied in rodents. Chronic kidney disease has been modeled by subjecting rodents to diets composed of high adenine, warfarin, or by partial or complete ablation of the kidneys, while calcification in chronic kidney disease models has been induced through diets high on vitamin D or phosphate.[212-214] These dietary and surgical provocations typically result in increased plasma concentrations of urea, phosphate, and FGF23 and cause extensive, though variable, medial vascular calcification, closely mimicking the reduced penetrance of vascular calcification–associated chronic kidney disease.

Decreasing circulating phosphate levels, attempted in a tumoral calcinosis mouse model, was also investigated in rodent models of chronic kidney disease. Nicotinamide prevents the development of hyperphosphatemia by suppressing intestinal sodium-dependent phosphate transport in rats with adenine-induced renal failure[215]; however its effect on ectopic calcification was not evaluated. Studies have shown that administering exogenous pyrophosphate via subcutaneous injection or continuous intraperitoneal infusion inhibits medial arterial calcification in vitamin D–toxic rats, without affecting bone formation or mineralization.[216-218]

Bisphosphonates have also been shown to prevent aortic calcification in uremic rats.[17] However, as in other vascular calcification disorders, such as GACI and PXE, attempts to treat chronic kidney disease–induced vascular calcification with bisphosphonates required doses that disrupted bone mineralization and architecture.[17] Additionally, the bone toxicity coupled with the fact that bisphosphonates require renal clearance has discouraged their use in chronic kidney disease.[17]

Low-density lipoprotein receptor–null mice fed a high-fat and -cholesterol diet develop characteristics of metabolic syndrome, including hypertension, obesity, dyslipidemia, and insulin resistance, in addition to robust vascular calcification with some evidence of cartilaginous metaplasia.[219,220]

Mouse models recapitulating disorders of early aging also present with calcification. *Lmna*-knockin mice recapitulate some of the clinical manifestations of Hutchinson–Gilford progeria syndrome, an accelerated aging disorder that leads to premature death.[221] *Lmna*-mutant mice show excessive aortic calcification. Vascular smooth muscle cells derived from these animals have increased TNAP expression and activity, subsequently reduced pyrophosphate levels, and therefore a reduced capacity to inhibit in vitro calcification.[221] Treatment of these mice with intraperitoneal injections of pyrophosphate inhibited vascular calcification.[221] It has been questioned, however, if the rapid hydrolysis of pyrophosphate in vivo prevents translation of this therapy to patients.[222]

4 DISEASE MECHANISMS

4.1 Regulators of Calcification: Phosphate, Pyrophosphate, and Matrix Gla Protein

Mineralization of cartilage, bone, and tooth extracellular matrix is a physiological process, whereas ectopic calcification is a pathological one. Nevertheless, evidence suggests that ectopic calcification, like bone formation, is a highly regulated process involving both inductive and inhibitory processes. These determinants include extracellular levels of calcium, the presence of a scaffolding extracellular matrix for mineral deposition, and the relative amounts of mineralization activators (e.g., inorganic phosphate) and inhibitors (e.g., pyrophosphate and matrix Gla protein) present within the extracellular matrix environment. Fetuin, osteopontin, and osteoprotegerin are also negative regulators of calcification; although

data suggest that they are more actively involved in atherosclerosis and bone mineralization rather than ectopic calcification, and will not be discussed further.[223] These factors are tightly balanced both in the circulation and in local microenvironments to maintain homeostasis and prevent pathologic calcification.

4.2 Phosphate

Inorganic phosphate is a major component and promoter of calcification. In the presence of calcium, various calcium phosphate salts are formed to neutralize the negative inorganic phosphate ions; these amorphous calcium phosphate precipitates eventually form hydroxyapatite.[224,225] In culture systems, high phosphate can induce osteochondrogenesis (see Section 4.6). In more common disorders of vascular calcification, serum phosphate is elevated, especially in patients with chronic kidney disease.[226]

The role of phosphate in pathologic calcification is most readily appreciated in patients with tumoral calcinosis, whose hyperphosphatemia leads to precipitation of phosphate and calcium in ectopic tissue. GALNT3, KLOTHO, and FGF23 are part of a tightly regulated phosphate reabsorptive pathway (Fig. 32.5). In the bone, GALNT3 O-glycosylates FGF23, preventing the proteolytic processing of FGF23, and therefore allowing the secretion of intact FGF23 into the circulation. In the kidney, FGF23 binds to the FGFR1 receptor along with the coreceptor KLOTHO. FGF23 signaling inhibits expression of renal 1α-hydroxylase, thereby decreasing serum concentrations of 1,25-dihydroxyvitamin D and reducing intestinal inorganic phosphate absorption. In addition, FGF23 signaling downregulates the renal type 2 sodium–phosphate cotransporters (NaPi-2a and NaPi-2c), thereby decreasing renal tubular reabsorption of inorganic phosphate.[100] When this pathway is defective, there is increased phosphate reabsorption from both the gut and the kidneys.

The regulation of inorganic phosphate is also seen in IBGC. SLC20A2 is a sodium-dependent phosphate importer, while XPR1 is a phosphate exporter[227,228] (Fig. 32.8). Inactivating mutations in both genes cause IBGC, emphasizing the tight regulation of phosphate homeostasis. *XPR1* mutation–mediated calcium phosphate precipitation has been suggested to occur intracellularly, whereas mutations in *SLC20A2* are thought to lead to deposition of calcium phosphate in the extracellular matrix. However, this paradigm has yet to be established in vivo.[228]

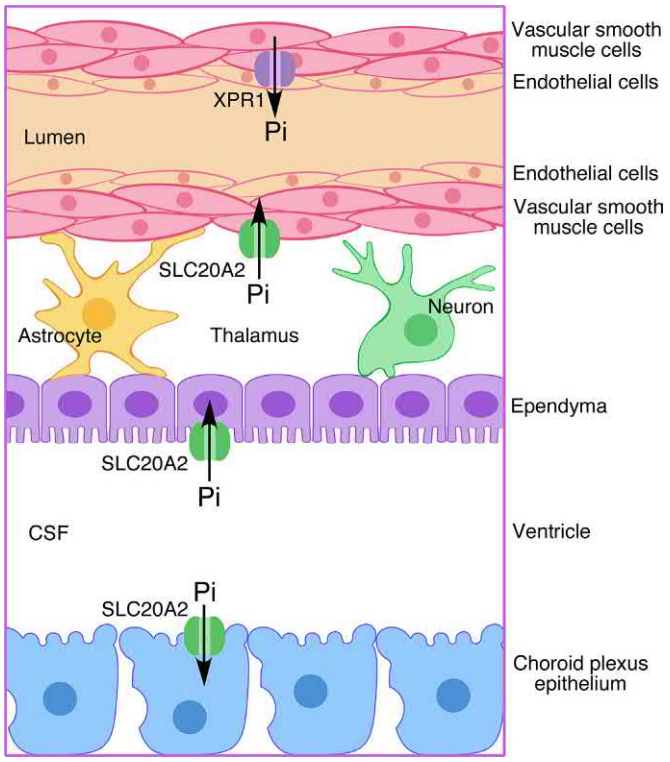

FIGURE 32.8 The regulation of phosphate in IBGC. SLC20A2, a sodium-dependent phosphate importer, is predominantly expressed on cerebrospinal fluid (CSF)–producing cells surrounding the ventricle and on vascular smooth muscle cells encompassing vessels in the brain. XPR1, a phosphate exporter, is also expressed on vascular smooth muscle cells. Inactivating mutations in *SLC20A2* and *XPR1* result in basal ganglia calcification emphasizing the tight regulation of phosphate homeostasis. While mutations in *SLC20A2* lead to increased cerebrospinal fluid phosphate levels and dysfunction of local vascular smooth muscle cells, which likely cause ectopic calcification in the extracellular matrix, *XPR1* mutation–mediated calcium phosphate precipitation has been suggested to occur intracellularly.

4.3 Pyrophosphate

Pyrophosphate acts as a potent inhibitor of calcification; it antagonizes the ability of inorganic phosphate to crystallize with calcium to form hydroxyapatite, presumably by occupying some of the inorganic phosphate sites on the surface of nascent growing hydroxyapatite crystals; the irregularities created slow down or terminate crystal growth.[229] Three molecules (ENPP1, ANK, and TNAP) have been identified as central regulators of pyrophosphate levels (Fig. 32.9).

ENPP1 is the primary source of extracellular PPi and hydrolyzes extracellular ATP into AMP and PPi.[230] ENPP1 is a cell surface glycoprotein enzyme that functions in synergy with the multiple-pass transmembrane protein ANK, which mediates intracellular-to-extracellular channeling of pyrophosphate.[168] While ENPP1 is known to work extracellularly, it has been recently shown that soluble ENPP1 functions intracellularly and might also be the source of pyrophosphate for transport by ANK.[172–174]

The extracellular concentration of pyrophosphate is further influenced by TNAP, another cell surface enzyme located on the membrane of cells and matrix vesicles. TNAP exerts its effects by hydrolyzing pyrophosphate, reducing

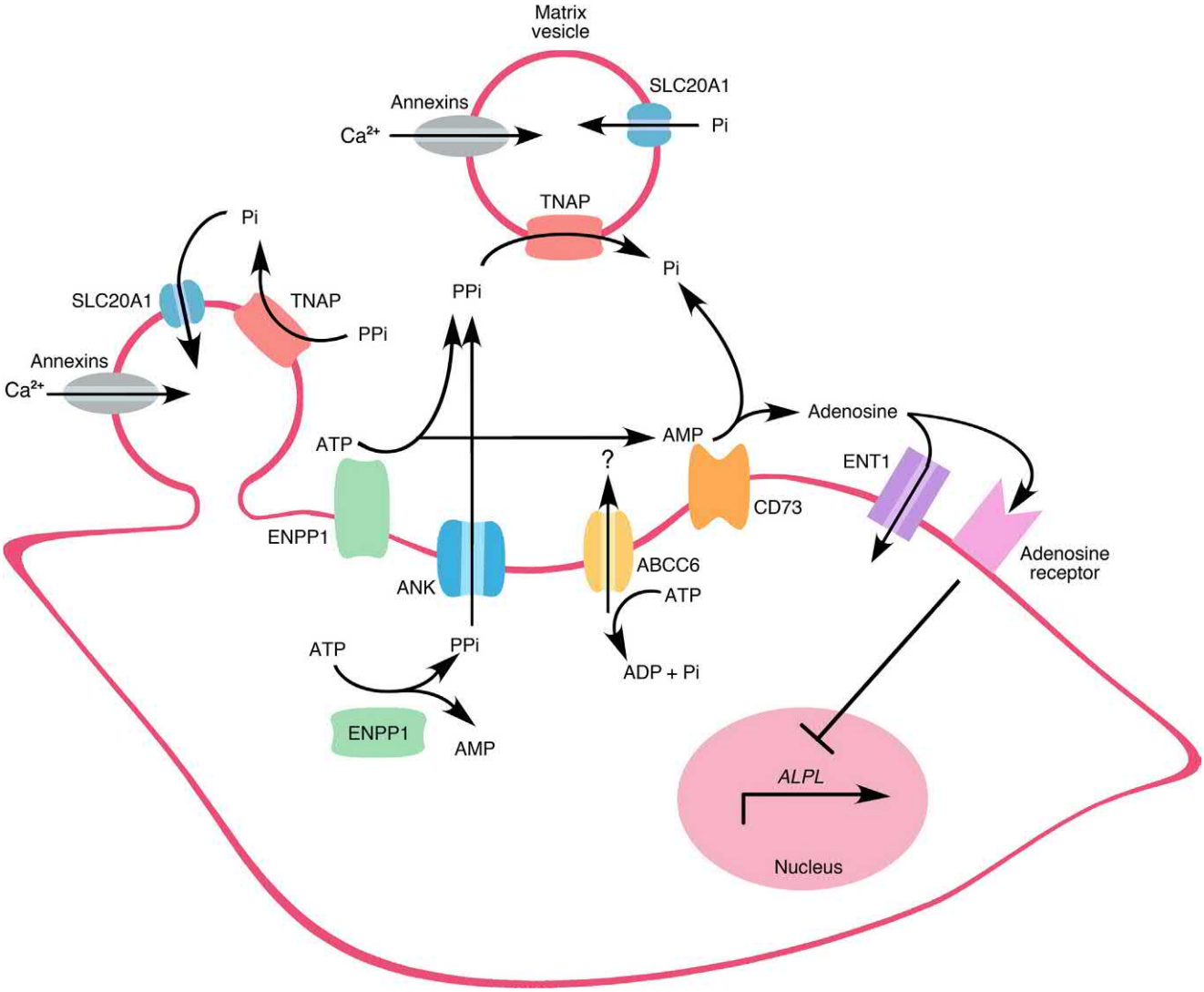

FIGURE 32.9 Schematic representation of the predominant enzymatic reactions and transported substrates in local cells involved in ectopic calcification. ENPP1 breaks down ATP into AMP and pyrophosphate. ANK transports pyrophosphate out of the cell, also contributing to extracellular pyrophosphate levels. CD73 further degrades AMP into adenosine and inorganic phosphate. ABCC6 is integral to the extracellular ATP metabolism pathway, though its substrate and function remain elusive. Adenosine can be transported into the cell by ENT1 or bind to a cell surface adenosine receptor to promote a number of diverse downstream actions, including repression of *ALPL*, the gene encoding tissue nonspecific alkaline phosphatase (TNAP). TNAP degrades pyrophosphate into inorganic phosphate and is a primary distal regulator of inorganic phosphate and pyrophosphate homeostasis. Matrix vesicles bud off of local cells and accumulate inorganic phosphate through SLC20A1 and calcium though annexins. Calcium and phosphate precipitate as a nidus of calcification inside the matrix vesicles. Continued calcium phosphate accumulation eventually leads to matrix vesicle breakage and propagation of hydroxyapatite in the extracellular space.

the concentration of this mineralization inhibitor, and establishing an inorganic phosphate/pyrophosphate ratio permissive for the formation of hydroxyapatite crystals.

Decreases in pyrophosphate cause pathologic calcification, as in GACI patients, reported to have low plasma[231] and urinary[232] pyrophosphate levels. While calcification in GACI appears directly related to pyrophosphate deficiency, the vascular mineralization in ACDC patients involves a downstream metabolite, namely adenosine. Adenosine signaling trophically inhibits *ALPL* expression.[20,175] Impaired intracellular signaling mediated by adenosine receptors is considered responsible for the increased levels of TNAP in ACDC patient–cultured fibroblasts.[20] Increased TNAP activity degrades extracellular pyrophosphate, promoting pathologic calcification.

Levels of pyrophosphate are reduced in hemodialysis patients[233] and correlate inversely with the amount of vascular calcification in patients with advanced chronic kidney disease.[234] Circulating pyrophosphate levels are primarily reduced, most likely because of increased alkaline phosphatase activity in the vessel wall. Consistent with these findings, intact aortas and aortic homogenates from uremic rats showed a substantial increase in TNAP enzyme activity.[235]

Pyrophosphate is considered a potent inhibitor of calcium phosphate crystal formation, but when present at high levels, pyrophosphate itself can precipitate with calcium ions to form an alternative type of crystal called CPPD. Indeed, gain-of-function mutations in *ANKH* cause chondrocalcinosis, and lead to higher steady-state concentration of pyrophosphate in the joint space.[82]

4.4 Matrix Gla Protein

Matrix Gla protein is recognized as a potent local inhibitor of vascular calcification.[140,236] To be fully functional, matrix Gla protein requires posttranslational modification by a γ-carboxylase, a vitamin K–dependent protein that is inhibited by warfarin. Interestingly, only when matrix Gla protein is overexpressed in vascular smooth muscles cells—instead of the liver—does it inhibit calcification in vivo, strongly suggesting that matrix Gla protein acts locally to prevent ectopic calcification.[237]

Matrix Gla protein binds to calcium and is secreted by chondrocytes and vascular smooth muscle cells.[206] It directly inhibits calcification, colocalizing with elastin in the arterial elastic lamina and physically disrupting calcium phosphate deposition.[238] Matrix Gla protein also sequesters bone morphogenic protein (BMP), specifically BMP2, in vitro and subsequently inhibits vascular BMP signaling and osteogenic differentiation[239]; it is unclear if this process is relevant in vivo.

4.5 Local Versus Circulating Hypotheses

Inorganic phosphate, pyrophosphate, and matrix Gla protein have been widely accepted as regulators of ectopic calcification, largely because they also function in the physiologic mineralization of skeletal hard tissues. There is considerable controversy, however, concerning the relative contribution of these factors from the local microenvironment versus the circulation. Determining the biologically relevant tissues and/or cell types producing these factors has been most extensively explored in the context of disease states, specifically PXE and IBGC.

The protein defective in PXE, ABCC6, is a member of the multidrug-resistant protein family, with known transporter activity,[240] but its endogenous substrate remains elusive. The ABCC6 protein has very low expression in the peripheral cells directly affected in PXE, that is, dermal fibroblasts and vascular smooth muscle cells,[241] but strong expression in the liver[242] and, to a lesser extent, kidney. This has led to the prevailing hypothesis that ABCC6 exports an endocrine inhibitor of calcification that acts at distant target sites,[243] but only circumstantial evidence exists for this "metabolic" theory (Fig. 32.10). Specifically, a wild-type muzzle transplanted onto the back of an *Abcc6*-mutant mouse developed

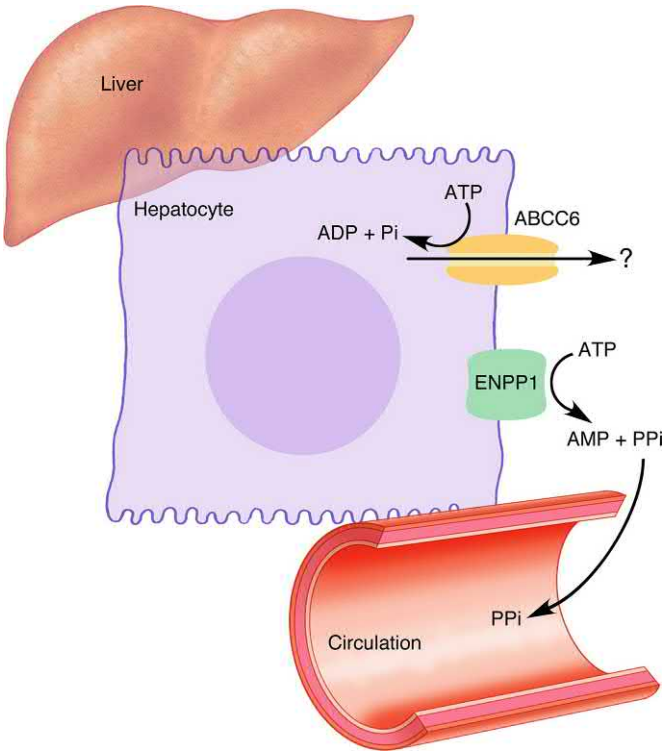

FIGURE 32.10 The contribution of circulating factors in PXE pathogenesis. The lack of knowledge pertaining to the substrate transported by ABCC6 complicates our understanding of PXE. The strong expression of ABCC6 in the liver has led to the prevailing hypothesis that hepatocellular ABCC6 exports an endocrine inhibitor of calcification that acts at distant target sites. However, recent evidence suggests that local cells might be involved in PXE pathogenesis. It has been shown that PXE patients and mice have decreased circulating pyrophosphate levels. Low pyrophosphate could be indirectly mediated by the actions of ENPP1 in the liver, or as a consequence of high TNAP activity in local cells. A unifying hypothesis would propose that mutations in *ABCC6* make local cells vulnerable to decreased circulating pyrophosphate.

ectopic calcification in the fibrous capsule surrounding the vibrissae.[244] It is unclear if the observed calcification completely recapitulates the PXE mouse model phenotype, as the extent of calcification of an *Abcc6*-mutant muzzle transplanted onto an *Abcc6*-knockout mouse was not investigated. In another study, parabiosis between *Abcc6*-mutant and wild-type mice in which the two circulations were connected showed attenuation of the calcification phenotype in the mutant mouse compared to that seen in the parabiosis of two *Abcc6*-mutant mice.[245] If PXE is solely driven by a circulating factor, however, it would be expected that both the mutant mouse and wild-type mouse would have the same degree of calcification, as they were paired before the onset of the phenotype; however, there was no calcification observed in the wild-type mouse when the *Abcc6*-mutant and control mice were paired. Taken together, these data suggest that a defect in a circulating factor is sufficient

to induce ectopic calcification and repletion of this factor diminishes the phenotype. However, as the *Abcc6*-mutant mouse in the *Abcc6*-mutant–wild-type mouse parabiosis pairing showed calcification, whereas its paired wild-type mouse did not, it is possible that another factor, perhaps a local cell, might also be involved in PXE-related calcification.

Dermal fibroblast cell lines derived from PXE patients are morphologically and biochemically distinct from controls; they have higher proliferation rates, decreased cell–cell and cell–matrix adhesion properties, and altered synthesis of connective tissue components, including elastin, collagen, and proteoglycans, in addition to different integrin subunit expression.[246,247] *ABCC6*-mutant cells also produce 30% less matrix Gla protein compared to controls.[248] Finally, *ABCC6*-mutant cells have a tendency for matrix mineralization and an altered metabolic profile, including higher gene expression of the PPi-metabolizing enzymes, namely TNAP[146,249].

Some scientists contend that these cultured fibroblast-specific changes are due to persistence of a circulating factor that impairs the function of the fibroblasts in vitro[250]; however, it is also possible that there are inherent, cell-autonomous defects in PXE patient–derived cells. Indeed *Abcc6*-mutant zebrafish showed signs of excessive pathologic calcification and *Abcc6* gene expression was localized to sites of ectopic calcification, specifically in osteoblasts, instead of the liver.[251]

The lack of knowledge pertaining to the substrate transported by ABCC6 complicates our understanding of PXE pathogenesis. It has recently been proposed that ABCC6 indirectly mediates ATP transport from the liver, which is then immediately broken down into AMP and pyrophosphate,[252] implicating pyrophosphate as the circulating metabolic factor missing in PXE (Fig. 32.10). In support of this hypothesis, it has been established that there are decreased levels of circulating pyrophosphate in PXE mice and humans. However, low levels of pyrophosphate could also be a result of high TNAP activity, which has been observed in *ABCC6*-deficient cells.[146]

A unifying hypothesis would propose that mutations in *ABCC6* make local cells (such as vascular smooth muscle cells or fibroblasts; Fig. 32.9) vulnerable to decreased circulating pyrophosphate, which is mediated by transport from the liver (Fig. 32.10). This hypothesis would reconcile the published results showing that there is low circulating pyrophosphate with the data demonstrating a cell-autonomous phenotype.

The dispute between circulating and local factors is also apparent in the disease pathogenesis underlying IBGC. *Slc20a2*-knockout mice have high inorganic phosphate levels in the cerebrospinal fluid compared to control mice[197] because of decreased phosphate reuptake in cerebrospinal fluid–generating tissues, including the choroid plexus and ependyma (Fig. 32.8). It has also been shown that *Slc20a2* is expressed locally in vascular smooth muscle cells, but not in pericytes or endothelial cells. When *Slc20a2*-deficient vascular smooth muscle cells are exposed to osteogenic media in vitro, they have an increased susceptibility to calcification. Thus, basal ganglia calcification is may be caused by the combination of abnormal cerebrospinal fluid phosphate homeostasis and increased susceptibility of vascular smooth muscle cells.[197]

These examples illustrate that both circulatory and local factors are necessary to maintain tissue homeostasis. In PXE, there is a circulatory deficit of blood pyrophosphate, whereas in IBGC there is excess inorganic phosphate in the cerebrospinal fluid. In both diseases, local cells are sensitized by their basic genetic defects to the circulating imbalances, resulting in pathologic calcification.

4.6 Osteochondrogenic Differentiation

While dysregulation of pyrophosphate and inorganic phosphate may directly lead to precipitation of calcium phosphate and eventual hydroxyapatite formation, local changes in pyrophosphate and inorganic phosphate homeostasis might also trigger cell-mediated mineralization, acceleration of the calcification process, and potential for ossification and/or chondrification. Transdifferentiation of vascular smooth muscle cells into osteochondrocyte-like cells has been reported in the media of calcified vessels of rare diseases (e.g., Keutel syndrome[139,206]) and common disorders, such as chronic kidney disease, type 2 diabetes, and patients on dialysis.[220,253-256] These cells express bone- and/or cartilage-specific proteins, such as TNAP, which often colocalize with calcium phosphate minerals in the vessel wall.[253] Matrix vesicles, which accumulate calcium and phosphate and eventually become the nidus for calcification, have also been identified in situ in these sites of calcification.[257]

In vitro, cells derived from the arterial media, mainly vascular smooth muscle cells, undergo osteochondrogenic differentiation in response to elevated inorganic phosphate levels by increasing expression of bone regulatory proteins, while simultaneously downregulating markers of smooth muscle lineage.[258,259] Specifically, when stimulated with inorganic phosphate, these cells express the sodium-dependent phosphate transporter (*SLC20A1*), which induces the early osteogenic/chondrogenic transcription factor RUNX2.[254,260] In addition to inorganic phosphate dysregulation, a number of other signaling pathways have been implicated in directly

promoting osteochondrogenic differentiation, including BMP2[261] and PDGF–BB signaling through PDGFRB.[262] These osteochondrocyte-like cells elaborate matrix vesicles[263] and eventually mineralize their extracellular matrix, much like the physiological conditions driving bone formation.[264]

There is conflicting evidence pertaining to the onset of lineage reprogramming in the context of pathologic calcification. Some evidence suggests that osteochondrogenic differentiation occurs before calcium deposition, such as in matrix Gla protein–null mice where calcified cartilaginous lesions originate from phenotypically transformed vascular smooth muscle cells.[264] However, other reports indicate that while cells with osteochondrocyte-like morphology are resident in the calcified aorta, osteochondrogenic markers are not upregulated in the arteries prior to the initiation of calcification.[265]

To probe the relative contributions of osteochondrogenic transdifferentiation versus calcium phosphate deposition, the *Mgp*-null mouse was independently bred with transglutaminase 2– and Elastin-knockout mice. Knocking out transglutaminase 2, a promoter of BMP signaling in matrix Gla protein–null mice decreased calcification, presumably by inhibiting the phenotypic transdifferentiation of vascular smooth muscle cells into osteochondrocyte-like cells.[266] Elastin haploinsufficiency in matrix Gla protein–null mice also significantly reduced arterial calcification.[265] Indeed, both genetic crosses reduced calcification, indicating that these processes are coexistent and likely necessary to promote ectopic calcification.

Despite the controversy over whether osteochondrogenic differentiation is an inciting event or a consequence of ectopic calcification, complete ossification or chondrification only occurs in a minority of diseased arteries. While bone and cartilage have been observed in vessels from mice with *Mgp* deficiency,[206] long-standing diabetes, and renal failure,[267,268.] bone or cartilage metaplasia has not been seen in any other disorders of ectopic calcification. This is possibly because of slower angiogenic invasion or the greater abundance of elastin, which maintains the smooth muscle cell phenotype.[269] Additionally, this cellular differentiation process might be attenuated in this disease context, maintaining only partial capacity for bone or cartilage formation.

4.7 Viral Mechanisms

There is also evidence that implicates the disruption of the viral maintenance machinery in ectopic calcification. MDA5, encoded by *IFIH1*, is a member of the RLR family and functions as a cytoplasmic pattern-recognition receptor, which recognizes viruses, double-stranded RNA, and secreted bacterial nucleic acids.[270] How these pathways intersect with the known pathways of ectopic calcification remains to be elucidated.

5 CONCLUSIONS

The balance between inorganic phosphate and pyrophosphate, in addition to other factors, is strictly controlled by a complex interplay of genes and plays an undisputed role in ectopic calcification. Understanding this homeostasis and the pathways involved will help us better identify new treatment targets and design therapeutic strategies. Insights into rare diseases should also inform more common presentations.

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SECTION 4

PARATHYROID AND RELATED DISORDERS

CHAPTER

33

Hyperparathyroidism

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Serum calcium levels are normally tightly controlled through regulated secretion of parathyroid hormone (PTH) by the parathyroid glands and its subsequent actions on bone, kidney, and intestine. Increases in serum calcium levels are sensed via the calcium-sensing receptor (CaR) on the surface of the parathyroid cell. When stimulated by an increase in the concentration of ionized Ca^{2+}, the CaR responds by activating secondary messengers to diminish PTH production and secretion. Over the short term, decreased CaR stimulation will increase secretion of PTH from granules stored in the cytoplasm and begin to increase transcription of the *PTH* gene and protein production. A prolonged absence of stimulus from CaR will trigger the cell to grow and divide.[1] Hypersecretion of PTH and parathyroid cell growth can therefore be secondary to an extrinsic, sustained demand for increased PTH or can be primary and independent of an external demand for PTH. Clinical disorders of PTH hyperfunctioning are characterized in these terms.

Secondary hyperparathyroidism is frequently associated with chronic kidney disease or vitamin D deficiency. If the underlying disease is corrected, the hyperparathyroidism will most often resolve. In some patients, however, especially in the setting of severe kidney disease, long-standing secondary hyperparathyroidism can progress to tertiary hyperparathyroidism in which PTH hypersecretion proceeds more independently of extrinsic factors, akin to primary hyperparathyroidism. Secondary and tertiary hyperparathyroidism will not be covered in detail in this chapter, mostly because the underlying acquired molecular genetic events that drive parathyroid tumor development in the latter have not yet been defined.

Primary hyperparathyroidism is a common clinical endocrinopathy, affecting as much as 0.1% of the population,[1] in which inadequately restrained PTH secretion results in elevation of serum calcium. The classical consequences of primary hyperparathyroidism are due to this elevation of serum calcium and include kidney stones, a bone resorptive phenotype, gastrointestinal disruption, and neuropsychiatric symptoms. However, most cases of primary hyperparathyroidism (particularly in the Western world) are identified by the incidental finding of elevated serum calcium during routine blood work. Thus, with advances in medical screening and increased access to healthcare, primary hyperparathyroidism has become a largely asymptomatic or minimally symptomatic disease.[2]

Neoplastic growth of one or more parathyroid glands is the cause of a large majority of all cases of primary hyperparathyroidism; although exceptions do exist, such as ectopic secretion of PTH by nonparathyroid tumors and heritable resetting of the calcium–PTH set point. Parathyroid neoplasia may be sporadic or familial, either isolated or syndromic. Benign, single-gland, parathyroid adenoma is the most common form of parathyroid neoplasia, accounting for about 85% of all cases. Diffuse hypercellularity, classically termed hyperplasia, of multiple parathyroid glands is observed in approximately 15% of cases. The benign forms of primary hyperparathyroidism typically, but not always, follow an asymptomatic or mildly symptomatic clinical course, which can be surgically cured with high success, or can sometimes be managed medically. Parathyroid carcinoma, in contrast, is a rare cause of primary hyperparathyroidism, but is often associated with severe clinical manifestations and significant mortality. A summary of the clinical characteristics of parathyroid adenoma and carcinoma is shown in Table 33.1.

Distinguishing between parathyroid adenoma and carcinoma is notoriously difficult on purely histopathologic grounds. Histopathologic features suggestive of

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TABLE 33.1 Clinical Characteristics of Parathyroid Adenoma and Carcinoma

Characteristics	Adenoma	Carcinoma
Female-to-male ratio	3.5:1	1:1
Average age (years)	55	48
PTH level	Mildly elevated	Markedly elevated
Asymptomatic patients (%)	80	<5
Patients with renal involvement (%)	4–18	32–80
Patients with skeletal involvement (%)	<5	34–91
Palpable neck mass	Rare	Common

PTH, Parathyroid hormone.
Adapted from Shane E. Clinical review 122: parathyroid carcinoma. J Clin Endocrinol Metab *2001;**86**(2):485.*[3]

malignancy, including fibrous bands and mitotic figures, may be occasionally be seen in the absence of unequivocal signs of invasion; of uncertain malignant potential, these tumors are referred to as atypical adenomas. A definitive diagnosis of carcinoma therefore depends upon the finding of a marked local invasion and/or regional or distant metastases already present at the time of surgery, thereby lowering the likelihood for surgical cure of established parathyroid malignancy.[4]

1 FAMILIAL HYPERPARATHYROIDISM

Primary hyperparathyroidism is seen as part of several familial tumor syndromes, including multiple endocrine neoplasia types 1, 2a, and 4 (MEN1, MEN2A, and MEN4); hyperparathyroidism–jaw tumor syndrome (HPT-JT); familial hypocalciuric hypercalcemia (FHH; also called familial benign hypercalcemia, FBH); neonatal severe hyperparathyroidism (NSHPT); and familial isolated hyperparathyroidism (FIHPT).[5] The MEN syndromes and FHH/NSHPT will be described in greater detail in Chapters 37 and 38. A summary of the genes implicated in familial tumor syndromes involving parathyroid neoplasia is shown in Table 33.2.

1.1 MEN1

MEN1 is a tumor predisposition syndrome involving multiple parathyroid, anterior pituitary, and enteropancreatic endocrine tumors (EPETs), with high penetrance. Tumorigenesis of other endocrine and nonendocrine tissues may also be seen.[6] Hypercalcemia is typically the first clinical manifestation of MEN1 and parathyroid tumors (generally in multiple glands) develop in approximately 95% of patients. The *MEN1* tumor suppressor gene was identified by positional cloning in kindred with linkage to chromosome 11q.[7] The overall frequency of coding region *MEN1* mutations in MEN1 patients is around 75%.[8–16] Germline mutations of *MEN1* are also responsible for a subset of families with FIHPT.

TABLE 33.2 Familial Tumor Syndromes Including Parathyroid Neoplasia

Syndromes	Associated genes	Tumor spectrum
MEN1/MEN4	*MEN1*[1,3,4] *CDKN1B*[2–4] *CDKN1A*[a,2,5] *CDKN2B*[a,2,5] *CDKN2C*[a,2–4]	Parathyroid, pituitary, EPETs, others[b]
MEN2A	*RET*[1,3,4]	Parathyroid, MTC, pheochromocytoma
HPT-JT	*HRPT2/CDC73*[1,3,5]	Parathyroid, fibroosseous jaw tumors, uterine, renal (hamartomas and Wilms)
FIHPT	*MEN1*[1,3,4] *HRPT2/CDC73*[1,3,5] *CASR*[1,4] *GCM2*[2,5] Others[c]	Parathyroid

EPETs, Enteropancreatic endocrine tumors; FIHPT, familial isolated hyperparathyroidism; HPT-JT, hyperparathyroidism–jaw tumor syndrome; MEN, multiple endocrine neoplasia; MTC, medullary thyroid cancer.
The criteria for inclusion of an associated gene were:
1. Clear evidence of Mendelian inheritance in families.
2. Association of gene variants with disease but inconclusive evidence of Mendelian inheritance (e.g., due to small family size).
3. Subject to recurrent clonal somatic mutation in parathyroid tumors.
4. Mutations functionally tumorigenic in a relevant in vivo model system.
5. Mutations affect protein function in a model system but tumorigenic role not yet demonstrated.

[a]*Germline mutations of* CDKN1A, CDKN2B, *and* CDKN2C *have been found in patients meeting the general criteria for MEN1.*
[b]*The full clinical spectrum of patients with germline* CDKN1B *(and other CDKI) mutations has yet to be established.*
[c]*As mutations/variants in the four stated genes are collectively found in only a minority of FIHPT kindred, additional genetic causes/contributors are strongly expected to exist.*

1.2 MEN2

MEN2 comprises three distinct variants, encompassing distinct tumor spectra, with one common genetic cause: germline-activating mutations of the *RET* oncogene.[17] *RET* encodes a transmembrane receptor tyrosine kinase that transduces intracellular signals in response to binding of certain growth factors, particularly of the glial cell line–derived neurotrophic factor (GDNF) family (reviewed in Ref. 18). Mutations within the extracellular domain can cause familial medullary thyroid cancer (MTC) alone, or MEN2A, which in addition to MTC, includes predisposition to pheochromocytomas and benign multigland parathyroid tumors. Mutations within the intracellular domain of RET predominantly cause MEN2B, which encompasses

MTC, pheochromocytoma, gastrointestinal ganglioneuroma, and mucosal neuromas, but almost never manifests primary hyperparathyroidism. Interestingly, loss-of-function *RET* mutations lead to an entirely different clinical disorder, Hirschsprung disease (megacolon with aganglionosis of the colon).[19] It has been hypothesized that the phenotype of specific *RET* mutations relates to ligand specificity.[20] Mutations in certain RET codons appear (634, 883, and 918) to correlate with more aggressive disease, and codon-specific management guidelines have been reviewed in Ref. 17.

1.3 MEN4

A MEN syndrome, with phenotypic similarities to both MEN1 and MEN2, dubbed MEN-X has been observed in a spontaneously occurring rat model. Affected rats develop parathyroid adenomas, pheochromocytomas, thyroid C-cell hyperplasia, paragangliomas, and EPETs.[21] The responsible gene was determined to be *Cdkn1b*, encoding cyclin-dependent kinase inhibitor (CDKI) $p27^{kip1}$. An 8-bp tandem duplication in exon 2, resulting in a frameshift mutation reducing p27 levels in vivo, was identified in affected animals.[22] This study also revealed a *CDKN1B* mutation in a patient with a personal history of pituitary adenoma and primary hyperparathyroidism, but who lacked any detectable *MEN1* mutation.[22] Subsequently, this syndrome of phenotypic MEN1 in patients with germline *CDKN1B* mutations was referred to as MEN4 (OMIM: #610755).

In additional studies, patients with MEN1 or clinical criteria suggestive thereof (e.g., multigland parathyroid hyperfunction or parathyroid plus another MEN1-related tumor), but lacking any detectable *MEN1* mutation, were screened for sequence abnormalities in *CDKN1B* and other CDKI genes. Additional mutations of suspected pathologic significance in *CDKN1B* were identified, as were sequence abnormalities in other genes encoding CDKIs (*CDKN1A*, encoding $p21^{Cip1}$; *CDKN2B*, encoding $p15^{Ink4b}$; and *CDKN2C*, encoding $p18^{Ink4c}$).[23] It is worth noting explicitly the confusing inconsistency in nomenclature caused by the syndromic naming of MEN4 when *CDKN1B* mutation is present, while maintaining the designation of MEN1 in instances when mutation in a different CDKI gene is present. Further studies are necessary to determine the complete clinical spectrum of patients with germline abnormalities of CDKI genes, but at this time it appears they collectively represent a small percentage of the subset of MEN1 cases that lack detectable mutations in the *MEN1* gene (reviewed in Refs. 24,25).

1.4 HPT-JT

While many families with primary hyperparathyroidism develop multigland parathyroid hyperplasia, a subset of families has demonstrated parathyroid adenomas, often with a cystic appearance on histopathologic examination, and occasionally parathyroid cancer. Parathyroid malignancy, while still uncommon, is markedly overrepresented in these families as compared with the general population. Often these families also demonstrate a predilection toward benign fibroosseous tumors of the jaw, histologically distinct from the giant cell "brown tumors" that have been associated with hyperparathyroidism, and less frequently a variety of kidney lesions, including renal hamartomas, kidney cysts, and Wilms tumor. An increased incidence of uterine tumors has also been noted.[26] HPT-JT is inherited in an autosomal dominant manner with high but incomplete penetrance. With the exception of the jaw, lesions in any of the target organs may be benign or malignant, and affected individuals may develop one or more lesions in any of the target organs over the course of their lifetimes.

Linkage studies excluded the other previously identified loci for familial hyperparathyroidism (chromosomes 10 and 11, the MEN2 and MEN1 loci, respectively) and established a novel familial hyperparathyroidism locus on chromosome 1q (HRPT2) for HPT-JT families.[27,28] The responsible gene, *HRPT2* (now renamed *CDC73*), was identified by positional cloning.[29] Coding region mutations have subsequently been identified in ~75% of established HPT-JT families and in a small subset of families in which hyperparathyroidism is the only phenotypic manifestation (reviewed in Refs. 30,31).

1.5 FIHPT

Primary hyperparathyroidism can also be seen as the only recurrent clinical manifestation within a family. Some cases of FIHPT are accounted for by mutations in *MEN1*,[32] *HRPT2*,[33] *CASR*, or CDKI genes. Recently, activating variants of the glial cells missing 2 (*GCM2*) gene have also been reported in FIHPT[34]; however, the role of these specific associated variants of undetermined penetrance in pathogenesis, or potentially in clinical management, has not yet been established. *GCM2* encodes an important parathyroid-specific transcription factor; inactivating *GCM2* mutations have previously been linked to hypoparathyroidism.[35] Linkage to a gene on chromosome 2p13.3-14 has been seen in several FIHPT pedigrees, but the responsible gene in these families has yet to be identified.[36] The underlying genetic defects in most families with FIHPT remain unknown.

1.6 FHH/NSHPT

Inactivating mutations of *CASR*, encoding CaR, are seen in patients with two types of FIHPT: FHH and NSHPT. Individuals with FHH inherit one abnormal

copy of the *CASR* gene, leading to asymptomatic hypercalcemia with normal or mildly elevated PTH levels. Despite these biochemical abnormalities, caused by affected cells' relative insensitivity to extracellular calcium, the parathyroid glands of these patients are generally normal in size, and treatment for the asymptomatic biochemical abnormalities is typically contraindicated. *CASR* mutation can also occasionally be expressed as FIHPT (see earlier), and the clinical spectrum associated with *CASR* mutations has been broadened to include, albeit rarely, symptomatic disease requiring treatment.[37]

NSHPT is mainly due to an individual's inheritance of two abnormal, inactivated copies of the *CASR* gene. Affected newborns exhibit severe hypercalcemia, skeletal abnormalities, and marked parathyroid hyperplasia, which is polyclonal in origin.[38] This condition can be fatal without parathyroidectomy.[39] FHH/NSHPT is discussed in more detail in Chapter 37.

2 SPORADIC HYPERPARATHYROIDISM

Insights into the molecular pathogenesis of sporadic parathyroid neoplasia have come from examination of acquired, clonal, tumor-specific, genetic lesions and also from the study of genes responsible for familial parathyroid neoplasia. Parathyroid adenomas are generally monoclonal and therefore represent true neoplastic outgrowths of a single abnormal cell. Parathyroid carcinomas are also monoclonal.[40] In addition, monoclonal parathyroid tumors are seen in familial MEN1[41,42] and in some tumors in the category of sporadic primary parathyroid hyperplasia.[43] The hypercellular parathyroid glands in patients with severe secondary/tertiary (uremic) hyperparathyroidism are often monoclonal in origin.[43]

Clonal tumor formation occurs through accumulation of genetic or genomic alterations that provide tumor cells with a growth advantage that is selected for in subsequent cell divisions, allowing the progeny of the progenitor cell to outcompete their neighbors. These somatic, tumor-driving events occur, by definition, in protooncogenes and tumor suppressor genes and affect the expression and/or function of the encoded gene products. The exact number and type of genetic alterations required for tumor formation are cell type and tumor type specific and may be modified by epigenetic events, environmental factors, and/or germline polymorphisms.[44] Some molecular genetic drivers of sporadic parathyroid tumors are known and it is expected that additional important alterations remain to be discovered. The genetic changes in benign parathyroid adenomas and malignant parathyroid carcinomas will be discussed separately here.

2.1 Parathyroid Adenoma

The most stringent criteria for genetic drivers of benign parathyroid tumorigenesis are: (1) the finding of recurrent, somatic mutations in typical, sporadically presenting but otherwise nonselected human parathyroid adenomas and (2) their ability to drive parathyroid tumorigenesis in experimental animal models (or in humans, if strong/Mendelian evidence of such a drive exists). Only two genes, *MEN1* and *CCND1* (encoding cyclin D1), solidly meet these criteria. *CDKN1B* and another cyclin-dependent kinase inhibitor (CDKI) are involved in predisposition to sporadically presenting (and familial) parathyroid tumors and appear to function rarely as genetic drivers of sporadic parathyroid tumorigenesis. Similarly, *CASR*, encoding the CaR, has a role in predisposition to parathyroid adenoma, but somatic mutations do not appear to drive sporadic parathyroid tumorigenesis. A number of additional genes have been reported as rare targets of somatic or germline mutation in benign, sporadic parathyroid adenomas, but their ability to drive parathyroid tumorigenesis has yet to be established experimentally.

In this discussion and previously analyzed series, we have defined typical sporadic adenoma as otherwise unselected cases with clinically determined single-gland disease, occurring in adults with neither a personal/family history of primary hyperparathyroidism nor a personal/family history suggestive of multiple endocrine neoplasia or a related syndrome, and for which neither gross examination nor histopathology demonstrates atypical or malignant features (described earlier). Defining criteria for case ascertainment is important, as use of different (especially less stringent) criteria in some studies may well influence the apparent genetic contributors that emerge.

2.1.1 MEN1

The *MEN1* tumor suppressor gene was first identified through its involvement in the familial MEN1 syndrome.[7] MEN1 is discussed more thoroughly in Chapter 38, but its relevance to sporadic parathyroid tumors is detailed here.

Allelic loss of chromosome 11q, the genomic location of the *MEN1* gene, is the most frequently detected genomic aberration in parathyroid adenomas. Following positional cloning of the *MEN1* gene in familial MEN1, somatic mutations of *MEN1* were identified in sporadic parathyroid tumors, with biallelic inactivation of *MEN1* occurring in 12%–35%.[45–50] Rarely, patients presenting with apparently sporadic parathyroid adenoma, perhaps subject to case ascertainment issues, may have germline *MEN1* mutations.[51]

Menin, the protein product of *MEN1*, contains no conserved structural domains with homology to other

known proteins, making ascertainment of the mechanisms by which it can suppress tumor growth more challenging. Menin is predominantly a nuclear protein[52] that can directly bind DNA.[53] Multiple potential binding partners of menin have been identified (reviewed in Ref. 54), suggesting that menin may have functions in regulation of transcription, genomic stability, cell division and proliferation, and epigenetic regulation. Menin can repress transcriptional activation mediated by transcription factor JunD,[55] regulating c-Jun–mediated transactivation of AP-1, a transcription factor with key roles in cellular proliferation and differentiation.[56] Menin interacts with SMAD3, a downstream component of the TGFβ signaling pathway; antisense menin provided cell with resistance to the growth inhibitory effects of TGFβ and inhibited the transcription of TGFβ target genes.[57] Menin also interacts with RPA2 and the FANCD2 protein, important proteins in DNA repair.[58] Menin appears to be a direct repressor of hTERT.[59] Menin has also been shown to directly interact with mixed lineage leukemia (MLL) family proteins as part of a histone methyltransferase complex that can activate transcription of CDKIs p27 and p18.[60] Menin also interacts with the DNA methyltransferase DNMT1, modulating the methylation status of p27 and p18 and antagonizing hedgehog signaling.[61] Loss of menin promotes DNMT1-mediated DNA hypermethylation and downregulation of Sox regulatory genes, thereby promoting Wnt/β-catenin signaling, increasing cell proliferation.[62] Thus, the documented binding partners of menin suggest important functional roles of menin, but none of the menin-binding partners or pathways in which menin is a part of have yet been proven to be essential to menin's tumor suppressive abilities in vivo.

To further characterize the role of menin loss in vivo, mouse models of MEN1 have been developed. Homozygous inactivation of *Men1* is embryonic lethal and mice heterozygous null for *Men1* develop a spectrum of tumors similar to the human syndrome.[63,64] Additional conditional knockouts of *Men1* have been developed using the Cre–LoxP system. Targeted inactivation of *Men1* specifically to the parathyroid glands resulted in parathyroid neoplasia accompanied by hypercalcemic hyperparathyroidism.[65]

2.1.2 *CCND1*

The finding of clonal rearrangements involving the PTH gene locus in a subset of parathyroid adenomas led to the identification of *CCND1* (*PRAD1*), encoding cyclin D1, as a human oncogene. This pericentromeric inversion of chromosome 11, resulting in the juxtaposition of the *PTH* 5′ regulatory region adjacent to the *CCND1*-coding region, places expression of *CCND1* under the control of the *PTH* promoter/enhancer (Fig. 33.1). The high level expression of *PTH* native to parathyroid cells results in a high-level overexpression of *CCND1* subsequent to *PTH–CCND1* rearrangement.[66] While rearrangement and/or translocation involving the *CCND1* locus occurs in up to 8% of sporadic parathyroid adenomas, overexpression of cyclin D1 has been seen in 20%–40% of these tumors.[67–69] Overexpression of cyclin D1 due to *CCND1* DNA amplifications or gene rearrangements have since been demonstrated in a variety of tumor types, confirming *CCND1*'s importance as a bona fide human oncogene.

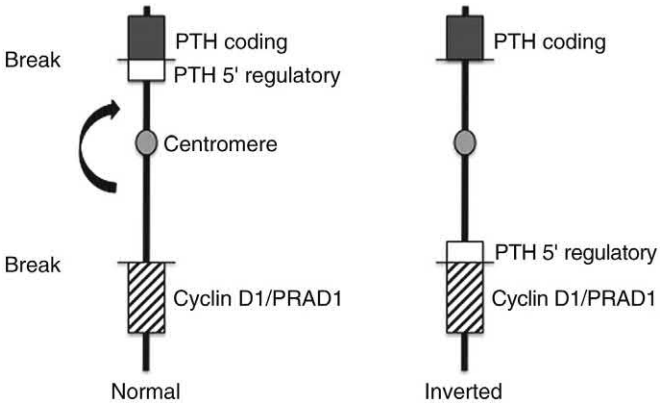

FIGURE 33.1 Schematic diagram illustrating the DNA rearrangement involving the PTH gene and the CCND1 gene in a subset of parathyroid adenomas. The chromosomal inversion event is deduced as the simplest cytogenetic event consistent with the molecular details of this DNA rearrangement. *Source: Figure adapted from Arnold A, Levine MA. Molecular basis of primary hyperparathyroidism. In: Bilezikian JP, editor.* The parathyroids. *3rd ed. San Diego, CA: Elsevier.*[70]

A mouse model of the *PTH–CCND1* rearrangement, similar to that found in human parathyroid tumors, has been developed.[71] In these transgenic mice, Cyclin D1 overexpression in the parathyroid glands is driven by the *PTH* regulatory region. These mice develop moderate chronic biochemical hyperparathyroidism and parathyroid gland hypercellularity, providing direct experimental evidence for cyclin D1's role as a driver of parathyroid neoplasia, and also establishing the mice as a model of human hyperparathyroidism.

While overexpression of cyclin D1 is a well-established driver of human tumorigenesis, the precise mechanisms through which cyclin D1–driven tumorigenesis occurs are controversial. The best characterized role of cyclin D1 is as a regulator of cell cycle progression. Cyclin D1 binds to, and activates, the cyclin-dependent kinases CDK4 or CDK6, which can then phosphorylate pRb, promoting G1–S phase transition. Cellular levels of cyclin D1 are regulated at multiple levels: in response to mitogenic signals, Ras signaling cascades result in increased cyclin D1 transcription and translation, and decreased nuclear export and cytoplasmic degradation, ultimately resulting in nuclear accumulation of cyclin D1. Dysfunction of any of these control mechanisms, resulting in increased nuclear cyclin D1, therefore can, and do,

contribute to tumorigenesis (reviewed in Ref. 72). More recently, CDK-independent functions of cyclin D1 have been described, which may have increased relevance to tumorigenesis. For example, overexpression of cyclin D1 has been shown to induce chromosomal instability, leading to genomic rearrangements and aneuploidy, which can promote tumorigenesis. Interestingly, a kinase-dead mutant form of cyclin D1 (cyclin $D1^{KE}$) induced a similar degree of aneuploidy as wild-type cyclin D1, and drove mammary tumorigenesis in transgenic mice with similar kinetics (reviewed in Ref. 73). It remains to be seen if similar, or other, kinase-independent functions of cyclin D1 can promote tumorigenesis in other tissues.

2.1.3 CDKN1B/CDKIs

A role for *CDKN1B* mutation in sporadic parathyroid tumors was suspected following the finding of its involvement in familial/syndromic hyperparathyroidism. Nonsynonymous, intragenic *CDKN1B* point mutations were identified in three patients with typically presenting sporadic parathyroid adenomas; in all three cases, the sequence variants were also present in the patients' germline DNA.[74] An identical c.397C>A (P133T) mutation, which was identified in two of the three patients, was subsequently identified as a germline variant in a sporadic parathyroid adenoma from an independent series.[75] One additional adenoma contained a somatic frameshift mutation coupled with tumor-specific loss of heterozygosity (LOH) at *CDKN1B* and the surrounding genomic region (Ref. 74 and unpublished observations). The scarcity of the identified variants in the global literature suggests they are highly unlikely to represent benign polymorphic variants distributed commonly in the general population. While LOH at the *CDKN1B* locus is uncommon in parathyroid tumors,[76,77] decreased p27 mRNA and protein expression has been described.[78–80] The unexpected finding of germline *CDKN1B* mutations in patients presenting with typical sporadic parathyroid adenomas in the absence of positive family histories and without early onset of the disease provides important evidence for the hypothesis that rare variants provide a genetic predisposition for common diseases, including primary hyperparathyroidism. This observation also raises the strong possibility that more rare variants will be identified as additional contributors to sporadic parathyroid adenoma development in the general population.

CDKN1B encodes $p27^{kip1}$, a cyclin-dependent kinase inhibitor involved in the G_1–S transition. The role of p27 in the cell cycle is complex. p27 binds to and inhibits active cyclin E–CDK2 and cyclin D–CDK4 complexes in the nucleus to aid in regulation of cell cycle progression at G_1.[81] Sequestration of p27 by active cyclin D–CDK4 complexes frees cyclin E/CDK2 complexes from inhibition, while reduction of cyclin D and/or CDK4 releases p27, allowing it to bind to and inhibit cyclin E/CDK2 complexes, arresting the cell cycle in G1. p27 is a known inhibitor of cyclin D1, an established parathyroid oncogene. Abundance and localization of p27 is highly controlled, primarily by posttranscriptional mechanisms, and decreased p27 levels and cytoplasmic localization have been correlated with tumorigenesis.[82] Aberrant expression of p27, often correlating with prognosis and/or therapeutic response, has been described in a variety of human tumors (reviewed in Ref. 83). Somatic mutations of *CDKN1B* are infrequent, but have occasionally been described in other tumor types, most notably small intestine neuroendocrine tumors.[84]

Several transgenic mouse models have been developed to examine the in vivo function of p27. Disruption of the cyclin–CDK inhibition domain of *Cdkn1b* resulted in enhanced growth and increased cell proliferation in tissues of homozygous null mice. Hyperplasia in the intermediate lobe of the pituitary was the most striking.[85] Similar results were observed by an independent, but similar, mouse model. Mice were larger than wild-type littermates. Disproportionate enlargement of the thymus, spleen, and pituitary was noted, and pituitary adenomas were found.[86] Nakayama et al.[87] also noted increased body size and multiorgan hyperplasia in their *Cdkn1b* knockout. Mice displayed a markedly enlarged thymus due to decreased apoptosis of thymocytes. Testicular, ovarian, and pituitary hyperplasia and pituitary adenomas were also seen. Bilateral cataracts due to disorganization of the neural retina were observed. Retinal abnormalities included: irregularity of outer limiting membrane, invasion of the rod and cone layer by the outer granular layer, displacement of bipolar cells within the inner granular layer, and increased numbers of amacrine cells (astrocyte like) and Muller's supporting cells.[87]

Subsequent mutational analysis of the remaining six CDKI genes, *CDKN1A*, *CDKN1C*, *CDKN2A*, *CDKN2B*, *CDKN2C*, and *CDKN2D*, encoding p21, p57, $p14^{ARF}$/p16, p15, p18, and p19, respectively, was performed in sporadic parathyroid adenomas. Nonsynonymous, intragenic point mutations in *CDKN1A*, *CDKN2B*, or *CDKN2C* were identified in five tumors; a single, somatic mutation was identified in *CDKN2C*, while the remaining mutations were germline or of undetermined germline/somatic status.[74] Two additional somatic frameshift mutations in *CDKN2C*, encoding p18, and two somatic missense variants in *CDKN2D*, encoding p19, were identified in another study.[88] No mutations of potential pathogenic significance were identified in *CDKN1C* or *CDKN2A*. Functional evidence supporting the potential pathogenicity of observed sequence variants has been demonstrated for five of the nine variants, representing three of the four genes (*CDKN1A*, *CDKN2B*, and *CDKN2C*).[74,88] Hypermethylation of *CDKN2A* and *CDKN2C*

has also been described in parathyroid tumors.[89] Mice null for *Ckdn2c*, encoding p18, rarely develop parathyroid neoplasia, but when crossed with *Cdkn1b*-null mice, p18/p27-null animals demonstrate an increased incidence of parathyroid tumors.[90] p18-null/Men1 heterozygotes also demonstrate an increased incidence of parathyroid tumors as compared to single knockout of p18 or *Men1* heterozygous littermates.[91] Thus, the evidence that the mentioned mutations/variants *CDKN1A, CDKN2B*, and *CDKN2C* may contribute to parathyroid tumorigenesis by somatic mutation or germline predisposition is strengthened by their ability, at least in combination, to drive parathyroid tumorigenesis experimentally.

2.1.4 β-*Catenin*

The contribution of Wnt signaling to parathyroid tumorigenesis has been examined, primarily through mutational and expression analyses of *CTNNB1*, encoding the oncogene β-catenin. Phosphorylation of β-catenin by GSK3β normally leads to its proteosomal degradation.[92] Mutation of *CTNNB1*'s GSK3β recognition motif (encoded by exon 3) can also lead to stabilization and accumulation of nonphosphorylated β-catenin, leading to increased activation of Wnt signaling.[93] Indeed, virtually all *CTNNB1* mutations identified in human tumors are located in exon 3 and most affect serine–threonine phosphorylation sites or adjacent residues, making this a hot spot for mutational activation of *CTNNB1* (reviewed in Ref. 94).

One center's studies of Swedish patients with parathyroid adenomas revealed an identical, somatic homozygous stabilizing mutation, encoding a serine-to-alanine change at amino acid 37 (S37A), in exon 3 of *CTNNB1*, in 9 of the 124 tumors studied. Aberrant β-catenin staining was observed in all tumors analyzed immunohistochemically, regardless of mutation status.[95,96] S37A mutation has not been identified in any additional patients in the nearly 600 additional parathyroid adenomas (including 98 from a distinct group of Swedish patients) collectively interrogated for mutations of *CTNNB1* exon 3 by other groups.[97–102] However, a heterozygous, somatic mutation encoding a serine-to-cysteine change at amino acid 33 (S33C) was identified in two patients from distinct cohorts,[103,104] suggesting *CTNNB*'s overall mutation frequency in parathyroid adenomas is likely less than 1.8% and perhaps as low as 0.3%. The two S33C mutations reported by other groups were heterozygous, also in contrast to the uniform homozygosity reported for the S37A mutation; heterozygosity would be more consistent with *CTNNB1*'s role as a direct-acting oncogene and with the copy number of such mutations when observed in other types of human tumors. Only 2 parathyroid adenomas of the 115 examined immunohistochemically in later studies, including only 1 of the 2 cases with S33C mutation, were reported to demonstrate abnormal β-catenin staining,[102,104] a value more consistent with the estimated mutation frequency. Aberrant splicing, and rarely somatic mutation, of the Wnt coreceptor LRP5, resulting in increased expression of β-catenin, has also been described in parathyroid adenoma.[50,104,105] The role of β-catenin and other Wnt signaling pathway components in parathyroid tumorigenesis is an important issue that merits further investigation.

2.1.5 *EZH2*

A heterozygous, somatic missense mutation of *EZH2*, an oncogenic contributor to multiple human tumor types, was identified in 1 of 8 tumors subjected to next-generation sequencing, and an identical mutation was found in 1 of 185 additional tumors examined by Sanger sequencing.[50] Identical mutation of *EZH2* has previously been described in follicular lymphoma and diffuse large–B cell lymphoma and has been demonstrated to act as a dominant, gain-of-function mutation.[106] Subsequent surveys of 23 and 82 typical parathyroid adenomas, respectively, failed to identify any additional *EZH2* mutations,[107,108] consistent with previous observations that *EZH2* Y641N mutations are uncommon, albeit potentially important, as candidate drivers of parathyroid neoplasia. *EZH2* gene amplification and overexpression of *EZH2* mRNA and/or EZH2 protein has also been reported in a subset of sporadic parathyroid adenomas.[109] While *EZH2* mutation and/or gene amplification has not yet been experimentally demonstrated to drive hyperparathyroidism, it is a strong candidate for rare involvement as a parathyroid oncogene.

2.1.6 *ZFX*

Recurrent, somatic mutations in *ZFX* were identified in 2 of 19 sporadic parathyroid adenomas subjected to whole-exome sequencing and an additional 4 tumors from an expanded cohort of 111 adenomas by Sanger sequencing.[110] Including one subsequent survey,[107] ZFX mutations have been sought and found in 3.9% of sporadic adenomas. The identified mutations were strikingly specific, affecting two adjacent arginine residues. Combined with documented expression of the mutant alleles within the tumor tissue, the observations suggest that mutant *ZFX* functions as a direct-acting oncogene in parathyroid adenoma.[110] *ZFX* encodes a transcription factor of the same name, with apparent roles in self-renewal of pluripotent stem cells,[111] and is overexpressed in a variety of tumor types.[112–116] *ZFX* has also been reported as a transcriptional target of the well-established parathyroid oncogene cyclin D1.[117] Further studies are required to directly show that mutant ZFX can drive parathyroid neoplasia, and to determine the precise mechanisms by which mutant *ZFX* contributes to parathyroid and potentially other types of human tumors.

2.1.7 CDC73

Following the identification of *CDC73* as the causative factor of the familial HPT-JT syndrome, in which the accompanying parathyroid adenomas often display a cystic appearance, several studies screened sporadic parathyroid adenomas for the presence of somatic *CDC73* mutations. When sporadically presenting adenomas are selected for cystic appearance,[29,118] large gland weight,[119] or clinical features (such as young age at presentation[51] or recurrence[120,121]), *CDC73* mutations are occasionally found (2.5%), and are most commonly germline, despite sporadic presentation of the disease. In otherwise unselected cases, somatic mutation of *CDC73* is very rare (1.5%),[33,122–125] in contrast to the much higher frequency detectable in sporadic parathyroid carcinoma.[122,123,125–127] Loss of parafibromin expression has also been described in a subset of parathyroid adenomas with cystic features.[128] The role of *CDC73*/parafibromin in parathyroid tumorigenesis is described in more detail in Section 2.2.

2.1.8 Calcium-Sensing Receptor

CaR, encoded by the *CASR* gene, on the surface of parathyroid cells monitors extracellular calcium levels, which, at least when chronically low, can serve as an important regulator of parathyroid cell growth. Calcium stimulates the G-protein–coupled CaR, which responds by activating phospholipase C, through G_q and G_{11}, resulting in production of inositol triphosphate and release of calcium from intracellular stores. Diacylglycerol concentrations also increase, stimulating protein kinase C, which phosphorylates CaR, promoting β-arrestin binding and internalization of CaR. CaR stimulation decreases PTH production and secretion. In the absence of a strong negative feedback stimulus from extracellular calcium, CaR is relaxed and PTH secretion is relatively unrestrained. Over the short term, a parathyroid cell lacking CaR stimulation will secrete PTH from granules stored in the cytoplasm and begin to increase transcription of the *PTH* gene and protein production. A prolonged absence of stimulus from CaR will trigger the cell to grow and divide.

Activating germline mutations of *CASR* are associated with a hypocalcemic, hypoparathyroid phenotype,[129] while inactivating mutations are seen in patients with FHH and NSHPT. Inactivating mutations result in reduced sensitivity to extracellular calcium, which alters the calcium–PTH set point in parathyroid cells. An increased level of Ca^{2+} is required to suppress PTH release. Mutations in genes involved in CaR signaling, *GNA11* (which encodes $G\alpha_{11}$) and *AP2S1* (which encodes AP2 σ) involved in CaR internalization, have also been demonstrated in FHH.[130,131] While parathyroid gland size and cellularity are at best minimally/mildly increased in FHH, severe parathyroid hyperplasia is seen in NSHPT along with increased serum calcium and PTH, suggesting that *CASR* could function as a parathyroid tumor suppressor gene.[132] Parathyroid hyperplasia has also been demonstrated in homozygous *Casr* knockout mice, but not in heterozygous knockout mice.[133] However, somatic, inactivating mutations have not been found in typical, sporadic parathyroid adenoma,[134,135] and must be quite rare if they exist at all. Germline *CASR* mutations have been described in sporadically presenting hyperparathyroidism,[51,136] although in only one case fitting our criteria for typically presenting, sporadic parathyroid adenoma,[136] underscoring the importance of case selection in genetic studies. Despite the rarity of mutations, aberrant *CASR* expression is more frequently seen in parathyroid tumors and may contribute at least to the hyperparathyroid biochemical phenotype, if not directly to parathyroid cell growth.[71]

2.1.9 Mitochondrial DNA

Mitochondrial alterations, including mitochondrial DNA (mtDNA) mutations have been described in a variety of tumor types (reviewed in Ref. 137). It has been hypothesized that a selective advantage conferred by mtDNA mutation could in particular contribute to benign tumorigenesis of a slowly replicating tissue, such as the human parathyroid. Acquired mtDNA mutations were identified in a subset of parathyroid adenomas, particularly in those with an oxyphil cell phenotype.[138] Oxyphil cells have a characteristic eosinophilic granular cytoplasm that is densely packed with mitochondria,[4,139] as compared with the typical chief cell. While the exact mechanism remains controversial and the concept still requires direct experimental validation in vivo, mtDNA mutations may well contribute to the molecular pathogenesis of benign parathyroid tumors. Statistically significant differences in mutation prevalence in oxyphil versus chief cell adenomas also suggest that mtDNA mutations may contribute to the oxyphil phenotype.[138]

2.1.10 Additional Genetic Aspects

A number of additional candidate genes, whose involvement in parathyroid tumorigenesis has been suspected, have also been examined. Benign parathyroid tumors are found in patients with germline *RET* mutations (MEN2A). However, somatic mutations of *RET* have not been identified in sporadic parathyroid adenomas.[140] It is unknown whether alterations in RET expression and/or function may contribute to the molecular pathogenesis of sporadic parathyroid tumors in some way.

Parathyroid cell proliferation is a normal response to vitamin D deficiency, in a manner similar to, and probably in large part mediated by, decreased serum calcium.

Clinically, patients with vitamin D deficiency or suboptimal vitamin D nutrition have increased parathyroid gland weight.[141] In parts of the world where profound vitamin D deficiency is endemic, severe and symptomatic primary hyperparathyroidism is more common.[142] However, vitamin D deficiency has only been directly correlated with more profoundly increased PTH levels in primary hyperparathyroidism, but was not associated with clinical manifestations, such as skeletal disease.[143] The PTH gene promoter contains a vitamin D responsive element (VDRE),[144] and $1,25(OH)_2D_3$ (the active form of vitamin D)–VDR complex suppresses PTH transcription and secretion.[145] Active vitamin D has been shown to suppress parathyroid cell growth both in vitro and in vivo.[146,147] In addition to its important role in parathyroid cell proliferation, vitamin D metabolism has been linked to tumorigenesis in various cell types. Active vitamin D can inhibit cell cycle progression, is associated with decreased cyclin D1 and increased p21 and p27 levels, and regulates growth factors, angiogenesis, apoptosis, and telomerase activity (reviewed in Refs. 148,149). Despite key roles in both parathyroid cell growth and human tumorigenesis, and reduced expression in parathyroid adenomas,[150,151] *VDR* mutations have not been found in parathyroid tumors.[152,153]

Rarely, germline alterations of the pituitary tumor predisposition gene, *AIP*, encoding the aryl hydrocarbon receptor–interacting protein, may be seen in sporadically presenting parathyroid adenomas. Moreover, 2 of 132 tumors screened for *AIP* mutations were positive for germline mutation, accompanied by loss of the normal allele in 1 case; *AIP* is located 2.6 Mb away from *MEN1* on 11q13, a location frequently subject to allelic loss in parathyroid adenomas. One of the two mutation-positive patients had persistent hypercalcemia/hyperparathyroidism following surgery, suggestive of multigland disease. The identical c.911G>A (R304Q) mutation was seen in both, unrelated patients and has been previously seen in several familial isolated pituitary adenoma kindred and sporadic pituitary tumors.[154] A germline *AIP* mutation has also been described in a single patient with a pituitary tumor and parathyroid hyperplasia, who tested negative for *MEN1* or *CDKN1B* gene mutations.[155] Homozygous inactivation of *Aip* in genetically engineered mice is embryonic lethal, and no parathyroid abnormalities have been reported in *Aip* heterozygous knockout mice.[156] In the absence of reported somatic mutations and direct experimental–functional evidence, it remains to be determined if *AIP* mutations can function as a genetic drivers of typical sporadic parathyroid adenoma; however, a rare predisposition allele of *AIP* is a good candidate for linkage to occasional cases of sporadic hyperparathyroidism.

Advances in sequencing technologies, allowing for analysis of virtually all transcribed exons throughout the entire genome, have been applied to sporadic parathyroid adenomas to a limited extent.[49,50] Interestingly, these two studies failed to identify any frequent genetic alterations in parathyroid adenomas or any alterations common to both studies (except *MEN1*), demonstrating the genetic heterogeneity of parathyroid adenomas. Mutations in a number of additional genes, such as *POT1*, were reported to affect single tumors, but could not be determined to be recurrent by Sanger sequencing of additional tumors. Further studies, including genetic and experimental–functional approaches, are required to determine the extent and nature of involvement of these additional genes in the pathogenesis of parathyroid adenomas.

2.1.11 Epigenetics

Epigenetic alterations in parathyroid adenomas have not been extensively studied, with only a few studies, focusing primarily on an individual gene or a small group of genes, having been performed. *RIZ1*, an Rb-interacting zinc finger gene, which appears to function as a tumor suppressor gene capable of driving tumor development in humans and experimental animals, was hypermethylated in 40% of the parathyroid adenomas studied; hypermethylation was accompanied by LOH at 1p36, the genomic locus of *RIZ1*.[157] However, aberrant expression of *RIZ1* has not been reported in parathyroid tumors and loss of *RIZ1* does not appear to be able to experimentally drive parathyroid tumor development in mice.[158] Hypermethylation of *APC*, the gene responsible for familial adenomatous polyposis, is a frequent finding in parathyroid adenomas.[159] Occasionally, parathyroid tumors have been seen in a familial adenomatous polyposis patients,[160,161] but owing to the relatively high prevalence of hyperparathyroidism, this may be a chance occurrence. Except in the setting of germline *APC* mutation,[160] aberrant *APC* expression has not been demonstrated in parathyroid adenomas,[99,162] but has been noted in parathyroid carcinomas.[99] *RASSF1A*[159] and *HIC1*[163] genes, frequently subject to epigenetic inactivation in human cancers, are also frequently hypermethylated in parathyroid adenomas. A comprehensive methylome analysis, including methylation sites of more than 14,000 genes, was performed on a series of benign and malignant parathyroid tumors. This study revealed aberrant methylation of 367 genes, including the previously identified targets *RIZ1*, *APC*, and *RASSF1A*, in parathyroid adenoma and 175 genes in parathyroid carcinoma, as compared to normal parathyroid glands. Methylation patterns of 263 genes differed between parathyroid adenoma and carcinoma.[89] Which of these aberrantly methylated genes are important in parathyroid tumor pathogenesis, and might ultimately play a role in developing novel therapeutic approaches, awaits further study.

2.2 Parathyroid Carcinoma

Parathyroid carcinoma is very rare but a highly aggressive form of primary hyperparathyroidism. The rarity of this tumor, further complicated by inconsistencies in diagnostic histopathologic criteria, has made the study of its genetic basis more difficult. There have been a few reports of carcinoma occurring within (and apparently evolving from) a hyperplastic or adenomatous parathyroid gland[164–168]; however, the disproportionately high prevalence of typical sporadic parathyroid adenoma compared with carcinoma implies that malignant progression from typical adenoma to carcinoma must be extremely rare.

Substantial evidence exists for a malignant progression model in a number of solid tumors, with normal tissue advancing through clinically apparent hyperplastic/ dysplastic and benign neoplasia stages, via incremental accumulation of acquired genetic abnormalities, before becoming malignant.[169] Whether parathyroid carcinomas follow such a clinically evident progression has been controversial. In a progression model, genetic alterations already present in early/benign disease are found at equal or greater frequencies in advanced/malignant disease, with additional alterations (that were important for progression) developing in the malignant tumors. For this progression model to hold true for parathyroid cancer, the same primary clonal genetic driver events already present in parathyroid adenomas should be at least equally represented in parathyroid carcinoma, along with any additional acquired genomic changes found in carcinomas. The most common alterations in benign parathyroid tumors, loss of 11q and accompanying mutation of *MEN1*, occur in 35% of parathyroid adenomas.[49,50,76,170–173] A progression model therefore would predict that 11q loss and/or *MEN1* mutation would be found in at least 35% of carcinomas; however, these changes are rarely, if ever, seen in parathyroid cancer.[170,171,173,174] These observations suggest that parathyroid cancer most commonly arises de novo, rather than evolving from a preexisting typical benign adenoma.[173]

2.2.1 CDC73

The setting of germline *CDC73* mutations, such as those seen in patients/families with HPT-JT or rarely FIHPT, is one exception to the aforementioned predominant process of de novo parathyroid carcinomagenesis. These patients indeed appear to develop parathyroid carcinomas that have evolved from preexisting benign or atypical adenomas, and likely explain some rare reports of apparent progression.

In contrast to their rarity in sporadically presenting benign parathyroid adenomas,[33,123,124] somatic, intragenic, inactivating mutations of *CDC73* are seen in a large percentage of malignant parathyroid tumors. Mutation frequencies of 67,[126] 100,[123] 86,[122] 13,[127] 60,[125] and 47%[175] have been noted across various studies. Discrepancies in mutation frequencies are not unexpected when cohort sizes are modest. They are also likely related, at least in part, to inconsistencies in inclusion criteria, given the well-known difficulties in solely histopathologic-based diagnosis of malignancy in a primary parathyroid tumor, in contrast to diagnosis based on the presence of clinically definitive features, such as recurrent/persistent hyperparathyroidism accompanied by gross local invasion or metastasis. For example, the histopathologic criteria for malignant vascular invasion may be particularly problematic; under the current World Health Organization (WHO) guidelines, only vascular invasion within or beyond the capsule meets this criterion, and intratumoral vasoinvasion alone is insufficient for a diagnosis of parathyroid carcinoma.[176] Immunohistochemical expression of the protein product of *CDC73*, parafibromin, is also lost in a majority of sporadic parathyroid carcinomas but retained in most sporadic adenomas. Owing to its relative specificity for parathyroid cancer, except in the setting of germline mutation, parafibromin immunohistochemistry has been proposed as an aid to the diagnosis of parathyroid cancer in clinically equivocal cases,[177–179] but parafibromin staining alone is not sufficient to serve as a diagnostic marker of parathyroid cancer.[180]

Importantly, a substantial minority of patients with seemingly sporadic parathyroid carcinoma possess germline *CDC73* mutations, suggesting they might represent new index cases of HPT-JT or a phenotypic variant, which has important implications for long-term management of these patients and their families.[122,126,181,182] A summary of *CDC73* mutations in both familial and sporadic hyperparathyroidism is shown in Fig. 33.2. Mutations identified to date are scattered throughout the 1593 coding base pairs of the 17 exon gene, with an as yet unexplained overrepresentation of mutations in exons 1, 2, and 7. In many tumors, biallelic *CDC73* inactivation can be demonstrated, through mutation accompanied by LOH or through independent mutations in both alleles,[122,123,126] as would be expected for a classical tumor suppressor gene. *CDC73* may also be inactivated through gross deletion of the gene.[118,183,184]

Parafibromin is a 531–amino acid, ubiquitously expressed, and evolutionarily conserved protein with a bipartite nuclear localization signal and is predominantly found in the nucleus. The C-terminal portion contains moderate sequence similarity to the yeast cell division protein Cdc73p, a component of the yeast polymerase-associated factor 1 complex (Paf1c), which associates with RNA polymerase II during transcriptional initiation and elongation. Additional evidence suggests Paf1c is involved in histone modification and posttranscriptional events, including modification of the poly(A) tail. The human PAF1 complex (hPAF1C) includes homo-

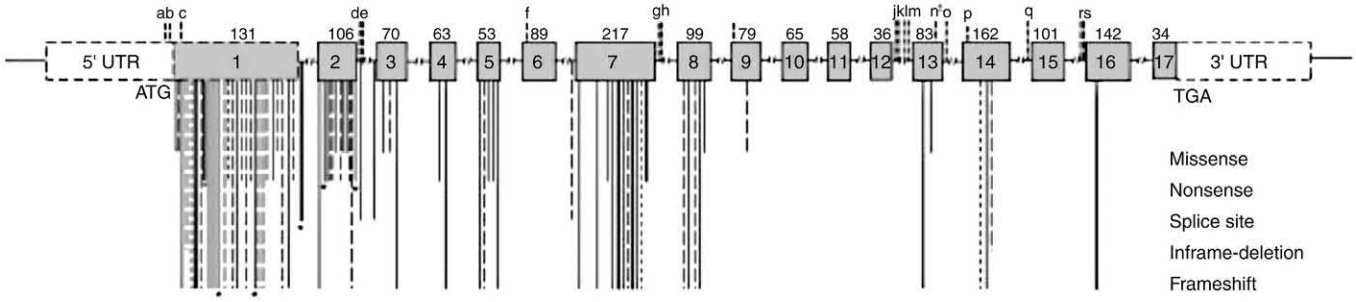

FIGURE 33.2 Schematic diagram of known mutations and polymorphisms in *CDC73*. The *CDC73* gene consists of 17 exons *(boxes)*, the coding region is indicated by *shaded boxes*, and the untranslated regions are *unshaded*. The sites of known mutations are indicated by *vertical lines* below the gene. The lengths of lines indicate different mutation types as illustrated (from shortest to longest: missense, nonsense, splice site, inframe deletion, and frameshift). Germline mutations *(solid lines)*, somatic mutations *(wide interrupted lines)*, and undefined mutations *(narrow interrupted)* are indicated. *Source: Figure originally from Newey PJ, Bowl MR, Cranston T, Thakker RV. Cell division cycle protein 73 homolog (CDC73) mutations in the hyperparathyroidism-jaw tumor syndrome (HPT-JT) and parathyroid tumors.* Hum Mutat *2010;***31***(3):295–307.*[31]

logs of most of the same subunits as the yeast Paf1c and shares similar functions.

Studies in *Drosophila* have demonstrated that Hyrax, the *Drosophila* homolog of parafibromin, is involved in canonical Wnt/Wingless signaling,[185] a central regulator of development and proliferation, and that parafibromin can rescue a Hyrax mutant phenotype. The involvement of parafibromin in canonical Wnt/β-catenin signaling provides one potential mechanism for its role in tumorigenesis; however, the precise contributions of parafibromin to Wnt signaling remain unclear. Activation of canonical Wnt signaling leads to activation of gene transcription by β-catenin; many targets of Wnt signaling promote cell proliferation. Expression of cyclin D1, an oncogene capable of driving parathyroid neoplasia, is regulated in part by Wnt signaling[186,187] and Wnt pathway abnormalities have been well documented in various types of human tumors (reviewed in Ref. 188). Loss of Wnt pathway components APC and GSK3β[99] and accumulation of β-catenin have also been described in parathyroid cancer.[162] Parafibromin can directly bind β-catenin[185] and may do so in a phosphorylation-dependent manner[189]; the resultant effects of parafibromin on Wnt signaling may be cell type specific.[185,189,190] Parafibromin also appears to directly interact with the SV40 large T antigen, and appears to have opposing effects on proliferation in cell lines expressing SV40 large T,[191] further complicating interpretation of in vitro functional analyses performed in the absence of readily-available parathyroid cell lines (or those from other HPT-JT–related tumors). Parafibromin has also been demonstrated to inhibit cancer cell growth and cause G1 phase arrest in vitro, in part through regulation of Cyclin D1.[192,193] Cytoplasmic parafibromin, which is less abundant, has been demonstrated to interact with cytoskeletal proteins[194] and p53 mRNA, facilitating its degradation, and modulating p53-mediated apoptosis.[195]

Conventional and conditional transgenic mouse knockouts of *Cdc73* have been developed to elucidate the in vivo function of parafibromin. Homozygous deletion of *Cdc73* is embryonic lethal by embryonic day 6.5 and controlled germline deletion of *Cdc73* at later stages of development leads to growth retardation, severe cachexia, and death within 20 days. Loss of parafibromin was associated with increased apoptosis in many tissues,[196] consistent with in vitro findings.[195,197] No parathyroid gland abnormalities were described.[196] These results indicate that *Cdc73* expression may be important for both embryonic development and survival of adult mice. It remains to be determined how loss of *Cdc73* expression promotes tumorigenesis in tissues, such as parathyroid and kidney in humans, and whether this phenotype can be recapitulated in the setting of a model organism.

2.2.2 *Additional Genetic Considerations*

A number of studies have sought to identify locations of allelic imbalance and/or interrogate candidate genes for sequence and/or expression abnormalities in parathyroid carcinoma. Recurrent regions of allelic loss likely to contain a key tumor suppressor gene(s) have been localized to chromosomes 1p, 3, 13q, and 14, by comparative genomic hybridization and molecular allelotyping. Recurrent regions of allelic gains likely to contain driver oncogene(s) are located on chromosomes 1q and 16.[170–174,198] A number of genes whose roles in the molecular pathogenesis of a variety of other tumor types are well established have also been examined in parathyroid tumors. Important human tumor suppressor genes (such as *p53*,[199,200] *pRb*, and *BRCA2*[201]) and oncogenes (such as *RAS*[202]) do not appear to be genetically altered in parathyroid tumors.[40,200,203]

Next-generation sequence analyses of parathyroid carcinoma have been performed in three studies.[175,204,205] Whole-genome analysis of a single parathyroid carcinoma and a recurrence from the same patient revealed somatic point mutations in 23 genes; of these, 15 were detected in both the primary tumor and the recurrence,

7 were found only in the recurrence, and 1 (*PIK3CA*) was found only in the primary tumor. Of particular interest, mutations were identified in *MLL2*, a putative tumor suppressor gene known to interact with *MEN1*; *mTOR*, a gene upstream of the parathyroid oncogene Cyclin D1; and *THRAP3*, a member of the SNARP complex that can regulate Cyclin D1 expression.[204] A mutation was also identified in *CDKN2C*/p18; interestingly affecting the same amino acid residue as a mutation previously identified in a parathyroid adenoma.[206] In another study, whole-exome sequence analysis of parathyroid carcinomas from 8 patients revealed novel, recurrent mutations of *PRUNE2* in two tumors, with mutations identified in 2 additional carcinomas screened by Sanger sequencing, for an overall mutation frequency of 18% (4/22) in this series.[205] *PRUNE2* functions to suppress Ras homolog family member A (RhoA) activity, resulting in suppression of oncogenic cellular transformation. Additional mutations were identified in several kinase genes potentially involved in cell migration and invasion, including *MAP3K11*, *JAK1*, and *RIOK3*.

A third study, the largest to date, involved a total of 24 patients. Whole-exome sequencing was performed on 10 sporadic parathyroid carcinomas and patient-matched normal controls, and combined with data from previously published cases[205] for additional analysis. Utilizing results from whole-exome sequencing, combined with a literature review, a targeted 16-gene (ParThy) panel was generated and used to test an additional 7 parathyroid carcinomas. This combined approach yielded novel insights into the pathogenesis of parathyroid carcinoma, revealing alterations of the PI3K/AKT/mTOR pathway in 21% of cases, and recurrently mutated genes *ADCK1*, *AKAP9*, *ZEB1*, and *FAT3*. The study also confirmed *CDC73* (mutated in 47%) as the most common driver gene in this disease, and *CCND1*/Cyclin D1 (amplified in 29%) as a key player in the development of parathyroid cancer.

The new and broader understanding of the genomic landscape of parathyroid carcinoma as uncovered in these next-generation sequence analyses opens the door to specific functional studies to elucidate their roles in pathogenesis and to their potential clinical use as therapeutic targets.[175]

3 ECTOPIC PTH PRODUCTION

The ectopic secretion of PTH by nonparathyroid tumors is an extremely rare cause of primary hyperparathyroidism. Hypercalcemia associated with malignancy of a variety of tumor types is common, and is commonly due to aberrant production of parathyroid hormone-related protein (PTHrP) by the tumor. With the development of modern immunoassays for PTH, which show no cross-reactivity with parathyroid hormone-related protein, only a few cases of true ectopic PTH production have been reported.

In only a small number of reported cancers has it been convincingly documented that PTH was being produced by the nonparathyroid tumor cells themselves[207–209]; these include reports involving small-cell carcinoma of lung, malignant neuroectodermal tumor, thymoma, clear-cell ovarian carcinoma, and pancreatic neuroendocrine tumor, among others. In two of these cases, a mechanism for the PTH production was also determined. In the ovarian carcinoma reported by Nussbaum et al.,[210] ectopic PTH production by the tumor was well documented, and the underlying molecular basis for aberrant expression of PTH was determined to be a rearrangement of the *PTH* gene with amplification.[210] In a case of severe hyperparathyroidism resulting from ectopic PTH production by a pancreatic neuroendocrine malignancy, a mechanism of transactivation of the *PTH* gene was suggested due to the hypomethylation of the *PTH* gene promoter in the tumor tissue.[211]

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CHAPTER

34

Hypoparathyroidism

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1 INTRODUCTION

Hypoparathyroidism is characterized by hypocalcemia and hyperphosphatemia, which are the result of a deficiency in parathyroid hormone (PTH) secretion or action (Table 34.1).[1,2] Hypoparathyroidism has been reported to have an estimated prevalence of 26–37 cases per 100,000 of the US population[1,3] and may result from agenesis [e.g., the DiGeorge syndrome (DGS)] or destruction of the parathyroid glands (e.g., following neck surgery, or in autoimmune diseases), from reduced secretion of PTH (e.g., neonatal hypocalcemia or hypomagnesemia), or resistance to PTH [which may occur as a primary disorder [e.g., pseudohypoparathyroidism(PHP)], or secondary to hypomagnesemia].[1,2,4] In addition, hypoparathyroidism may occur as an inherited disorder (Table 34.2) that may either be part of a complex congenital defect (e.g., the DGS), or as part of a pluriglandular autoimmune disorder, or as a solitary endocrinopathy, which has been referred to as *isolated or idiopathic* hypoparathyroidism (Fig. 34.1).[1,5] Although familial forms of hypoparathyroidism are rare, studies into these disorders have yielded pivotal insights into key genes regulating PTH secretion and the maintenance of extracellular calcium homeostasis. Indeed, the characterization of kindreds with isolated or syndromic forms of hypoparathyroidism have helped elucidate the molecular basis of extracellular calcium sensing by the parathyroid glands and identified *CASR* and *GNA11* as critical genes in this process, and also revealed the *TBX1*, *GCMB*, and *GATA3* genes to encode transcription factors mediating parathyroid gland development. This chapter will briefly review the clinical features of hypoparathyroidism, followed by a more detailed discussion of the genetics and molecular pathology of the inherited hypoparathyroid disorders. This chapter will also outline a clinical approach to the investigation of genetic causes of hypoparathyroidism and provide an overview of recent therapeutic advances. The pseudohypoparathyroid disorders are reviewed in Chapter 35.

2 CLINICAL AND DIAGNOSTIC ASPECTS

Patients with hypoparathyroidism may be asymptomatic or develop symptoms and signs associated with hypocalcemia and hyperphosphatemia. The clinical presentation of hypocalcemia ranges from an asymptomatic biochemical abnormality to a severe, life-threatening condition. Normal total serum calcium is 2.15–2.65 mmol/L and in mild hypocalcemia (serum calcium 2.00–2.15 mmol/L), patients may be asymptomatic. Those with more severe (serum calcium less than 1.9 mmol/L) and long-term hypocalcemia may develop: acute symptoms of neuromuscular irritability (e.g., paraesthesia, tetany, seizures, laryngospasm, or bronchospasm); subcapsular cataract; papilledema; and abnormal dentition.[2,4] Moreover, persistent hyperphosphatemia causes ectopic mineralization, and >70% of patients with idiopathic hypoparathyroidism have been reported to have calcifications within intracerebral regions, such as in the basal ganglia.[8] Patients with hypoparathyroidism may also have low levels of bone turnover and elevations in bone mineral density.[4,9] Furthermore, hypoparathyroidism may lead to neurocognitive disorders, such as anxiety, phobic anxiety, and a tendency to depression.[10,11] Indeed, hypoparathyroidism has been associated with significant reductions in quality of life measures, even in treated normocalcemic patients.[10,11] Investigations should be directed at confirming the presence of hypocalcemia and establishing the cause (Table 34.1). In hypoparathyroidism, serum calcium is low, phosphate is high, and PTH is inappropriately low or undetectable; renal function and concentrations

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TABLE 34.1 Causes of Hypoparathyroidism

- Low parathyroid hormone levels (hypoparathyroidism)
  - Parathyroid agenesis
    - Isolated or part of complex developmental anomaly (e.g., DGS)
  - Parathyroid destruction
    - Surgery[a]
    - Radiation
    - Infiltration by metastases or systemic disease (e.g., hemochromatosis, amyloidosis, sarcoidosis, Wilson's disease, thalassemia)
  - Autoimmune
    - Isolated
    - Polyglandular (type 1)[a]
  - Reduced parathyroid function (that is, parathyroid hormone secretion)
    - Parathyroid hormone gene defects
    - Hypomagnesemia[a]
    - Neonatal hypocalcemia (may be associated with maternal hypercalcemia)
    - Hungry bone disease (postparathyroidectomy)
    - Calcium-sensing receptor mutations
- High parathyroid hormone levels
  - Parathyroid hormone resistance (e.g., PHP, hypomagnesemia)

[a]*Most common causes.*
DGS, DiGeorge syndrome; PHP, pseudohypoparathyroidism.
Reproduced from Thakker RV. Hypocalcaemic disorders, hypoparathyroidism, and pseudohypoparathyroidism. In: Wass JAH and Stewart PF, editors. Oxford Textbook of Endocrinology and Metabolism, *2nd ed. Oxford: Oxford University Press; 2011. p. 675–86.*[7]

of the 25-hydroxy and 1,25-dihydroxy metabolites of vitamin D are normal.[1] In *PHP* these findings are similar to those of hypoparathyroidism except for PTH, which is markedly increased.[5]

3 COMPLEX SYNDROMES ASSOCIATED WITH HYPOPARATHYROIDISM

Hypoparathyroidism may occur as part of a complex syndrome, which may either be associated with a congenital developmental anomaly or with an autoimmune syndrome.[5] The congenital developmental anomalies associated with hypoparathyroidism include the DiGeorge, the hypoparathyroidism, deafness and renal anomalies (HDR), the Kenny–Caffey and the Barakat syndromes, and also syndromes associated with either lymphedema or dysmorphic features and growth failure (Table 34.2).

3.1 DiGeorge Syndrome

DGS comprises five major phenotypes, which are: cardiac outflow tract malformations, facial dysmorphia, palatal dysfunction, hypoparathyroidism, and immune deficiency related to thymic hypoplasia.[12] DGS has been reported in up to 60% of children with familial or idiopathic forms of hypoparathyroidism,[13] and has a wide spectrum of severity. Most commonly, hypoparathyroidism associated with DGS presents in the neonatal period with marked hypocalcemia, which may cause laryngospasm or seizures.[14] However, the hypoparathyroidism may be transient and characterized by serum calcium levels that normalize during infancy and early childhood. Some individuals with DGS may only develop hypocalcemic symptoms in adolescence or adulthood.[14,15] Moreover, DGS may be associated with a form of latent hypoparathyroidism characterized by normal serum calcium and PTH concentrations, but with an inability to increase PTH secretion in response to a hypocalcemic challenge.[16] The disorder arises from a congenital failure in the development of the derivatives of the 3rd and 4th pharyngeal pouches with resulting absence or hypoplasia of the parathyroids and thymus. Most cases of DGS are sporadic but an autosomal dominant inheritance of DGS has been observed and an association between the syndrome and an unbalanced translocation, and deletions involving 22q11.2 have also been reported,[17] and this is referred to as DGS type 1 (DGS1). Mapping studies of the DGS1 deleted region on chromosome 22q11.2 have defined a 250–3000 kb critical region that contained approximately 30 genes. Studies of DGS1 patients have reported deletions of several of the genes (e.g., *rnex40*, *nex2.2–nex 3*, *UDFIL*, and *TBX1*) from the critical region.[12,18] However, point mutations in DGS1 patients have only been detected in the *TBX1* gene,[19] and *TBX1* is now considered to be the gene causing DGS1.[12] TBX1 is a DNA binding transcriptional factor, of the T-Box family, that is known to have an important role in vertebrate and invertebrate organogenesis and pattern formation.[20–22] The TBX1 gene is deleted in ~96% of all DGS1 patients. Moreover, DNA sequence analysis of unrelated DGS1 patients who did not have deletions of chromosome 22q11.2 have revealed the occurrence of 3 heterozygous point mutations[19] and a 23-bp deletion.[23] One of the point mutations resulted in a frameshift with a premature truncation, while the other two were missense mutations (Phe148Tyr and Gly310Ser). All of these patients had the complete pharyngeal phenotype but did not have mental retardation or learning difficulties.[19]

In some patients, deletions of locus on chromosome 10p have been observed in association with DGS[24] and this is referred to as DGS type 2 (DGS2). Patients with DGS2 may have a more severe phenotype than DGS1, and present with marked cognitive impairment in addition to cardiac abnormalities and immune deficiency.[25,26] The nebulette (NEBL) gene has been reported to be heterozygous deleted in cell lines from two female DGS2 patients, and thus may be the responsible gene.[27] Moreover, some patients presenting with the clinical manifestations of DGS, such as thymic aplasia and hypoparathyroidism, may additionally have features of the CHARGE

TABLE 34.2 Inherited Forms of Hypoparathyroidism and Their Chromosomal Locations

Disease	Inheritance	Gene product	Chromosomal location
Isolated hypoparathyroidism	Autosomal dominant	PTH[a]	11p15
	Autosomal recessive	PTH,[a] GCMB	11p15, 6p24.2
	X-linked recessive	SOX3	Xq26-27
ADH Type 1	Autosomal dominant	CaSR	3q21.1
ADH Type 2	Autosomal dominant	$G\alpha_{11}$	19p13.3
Hypoparathyroidism associated with complex congenital syndromes			
DGS1	Autosomal dominant	TBX1	22q11.2
DGS2	Autosomal dominant	NEBL[b]	10p13-14
CHARGE syndrome	Autosomal dominant	CHD7	8q12.1
HDR	Autosomal dominant	GATA3	10p15
Hypoparathyroidism associated with Kearns–Sayre and MELAS	Maternal	Mitochondrial genome	—
MTP deficiency	Autosomal recessive	MTP β-subunit	2p23
Kenny–Caffey Type 1, Sanjad–Sakati	Autosomal recessive	TBCE	1q42.3
Kenny–Caffey Type 2	Autosomal dominant	FAM111A	11q12.1
Kirk–Richardson			
Dubowitz syndrome	Autosomal recessive	Unknown	?
Barakat	Autosomal recessive[b]	Unknown	?
Lymphedema	Autosomal recessive	Unknown	?
Nephropathy, nerve deafness	Autosomal dominant[b]	Unknown	?
Nerve deafness without renal dysplasia	Autosomal dominant	Unknown	?
Hypoparathyroidism (APECED)	Autosomal recessive	AIRE	21q22.3
PHP (type 1a), and pseudoPHP[c]	Autosomal dominant parentally imprinted	GNAS1	20q13.3
PHP (type 1b)[c]	Autosomal dominant parentally imprinted	GNAS1, NESP55, STX16	20q13.3

[a]*Mutations of PTH gene identified only in some families.*
[b]*Most likely gene (or inheritance) shown.*
[c]*Listed for completeness, but reviewed in Chapter 35.*
ADH, Autosomal dominant hypocalcemia; APECED, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome (also known as autoimmune polyglandular syndrome type 1); AIRE, Autoimmune regulator; CaSR, calcium-sensing receptor; DGS1; DiGeorge syndrome type 1; DGS2, DiGeorge syndrome type 2; HDR, hypoparathyroidism, deafness, and renal anomalies; MELAS, mitochondrial encephalopathy, stroke - like episodes and lactic acidosis; MTP, mitochondrial trifunctional protein; NEBL, nebulette; PTH, parathyroid hormone; PHP, pseudohypoparathyroidism; TBCE, tubulin-specific chaperone; ?, location not known.
Adapted from Thakker RV. Hypocalcaemic disorders, hypoparathyroidism, and pseudohypoparathyroidism. In: Wass JAH and Stewart PF, editors. Oxford Textbook of Endocrinology and Metabolism, *2nd ed.; 2011. Oxford: Oxford University Press, p. 675–86.*[7]

syndrome, which is characterized by the combined occurrence of Coloboma, Heart abnormalities, Choanal Atresia, Retardation of growth and/or development, and Genitourinary and/or Ear anomalies.[28] Such patients with overlapping features of DGS and the CHARGE syndrome have been revealed to harbor heterozygous mutations in the *CHD7* gene on chromosome 8q12.1, which encodes the chromodomain helicase DNA binding protein 7.[28] The *CHD7* gene is expressed within the pharyngeal ectoderm, and *Chd7* haploinsufficient mice have been noted to have thymic aplasia.[29] These findings highlight a role for CHD7 in pharyngeal region development during embryogenesis.

3.1.1 Mouse Models for DGS1

Transgenic mice with deletion of *Tbx1* have a phenotype that is similar to that of DGS1 patients.[20] Thus, *Tbx1* null mutant mice ($Tbx1^{-/-}$) had all the developmental anomalies of DGS1 (i.e., thymic and parathyroid hypoplasia; abnormal facial structures and cleft palate; skeletal defects; and cardiac outflow tract abnormalities), while *Tbx1* haploinsufficiency in mutant mice ($Tbx1^{+/-}$)

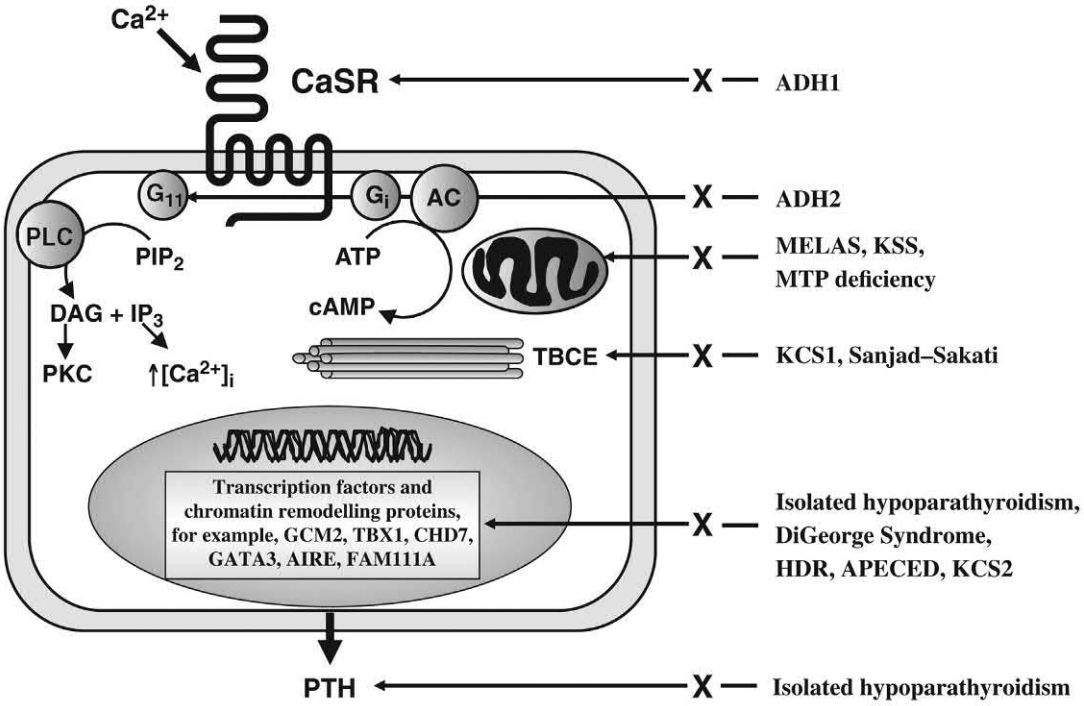

FIGURE 34.1 Schematic representation of some of the components involved in the parathyroid regulation of calcium homeostasis. Alterations in extracellular calcium are detected by the calcium-sensing receptor (CaSR), which is a 1078 amino acid G-protein coupled receptor. The CaSR signals via the G_{11} protein to stimulate phospholipase C (PLC), which catalyses the hydrolysis of phosphoinositide (PIP_2) to inositol triphosphate (IP_3), thereby increasing intracellular calcium, and diacylglycerol (DAG), which activates protein kinase C (PKC). The CaSR also signals via the G_i protein, which inhibits adenylate cyclase (AC), thereby leading to a reduction in the formation of cAMP from ATP. These proximal signals modulate downstream pathways, which lead to alterations in the synthesis and secretion of PTH. Abnormalities in several genes and encoded proteins in these pathways, have been identified in patients with hypoparathyroid disorders (Table 34.2). *Source: Adapted from Thakker RV. Parathyroid disorders: molecular genetics and physiology. In: Morris PJ, Wood WC, editors.* Oxford Textbook of Surgery, *Oxford University Press; 2000. p. 1121–9.*[6]

was associated only with defects of the 4th pharyngeal pouch (i.e., cardiac outflow tract abnormalities). *Tbx1* in mice is expressed in the pharyngeal ectoderm and endoderm, as well as in the mesodermal core of the pharyngeal arches, but is not expressed in neural crest cells[30] and the generation of several tissue-specific knock-out models of Tbx1 have revealed the following. Knockout of *Tbx1* in the pharyngeal endoderm led to neonatal death with malformations identical to $Tbx1^{-/-}$ mice, and likely due to failure of pharyngeal pouch outgrowth.[31] Inactivation of Tbx1 in the otic vesicle led to an absence of the inner ear.[32] Mesoderm specific knock-out of Tbx1 led to multiple phenotypes that included malformation of the inner ear, defective pharyngeal patterning, cardiovascular defects, defective development of the proximal mandible, and thyroid hypoplasia.[33–36] An "allelic series" of Tbx1 expression showed tissue-specific dosage effects, demonstrating that cardiac outflow tract development is more susceptible to loss of Tbx1 than craniofacial development.[37] Thus, these and other detailed studies in mice have revealed that inactivation of *Tbx1* results in abnormal early patterning and hypoplasia/aplasia of the pharyngeal arches, as well as impaired formation of the 2nd and 4th pharyngeal pouches.[20,38,39] Furthermore, inducible inactivation of *Tbx1* in conjunction with in vivo cell fate mapping has demonstrated that the early absence of *Tbx1* by embryonic day 7.5 (E7.5) resulted in a phenotype identical to that observed in germline $Tbx1^{-/-}$ knockout mice. Thus, inactivation of *Tbx1* at around E9.0 exclusively impaired the development of pharyngeal segments posterior to and including the 3rd pharyngeal pouch, thereby indicating that there is a likely anterior-to-posterior gradient of *Tbx1* activity over time and that *Tbx1* expression is tightly regulated in distinct segments during pharyngeal system development.[40] cDNA microarray analyses of mice lacking *Tbx1* have identified the transcription factor glial cells missing 2 (Gcm2) as one of the downregulated genes in the pharyngeal region,[41] thereby indicating that *Tbx1* is upstream of *Gcm2*. Moreover, Tbx1 is regulated by sonic hedgehog (Shh), thereby indicating a Shh-Tbx1-Gcm2 pathway in parathyroid development.[42,43] The basis of the phenotypic differences between DGS1 patients, who are heterozygous, and the $Tbx1^{+/-}$ mice remains to be elucidated. It is plausible that Tbx1 dosage, together with the downstream genes that are regulated by Tbx1 could provide an explanation, but the roles of these putative genes in DGS1 remains to be elucidated.

3.2 Hypoparathyroidism, Deafness, and Renal Anomalies Syndrome

The combined inheritance of HDR as an autosomal dominant trait was reported in one family in which patients had asymptomatic hypocalcemia with undetectable or inappropriately normal serum concentrations of PTH, and normal brisk increases in plasma cAMP in response to the infusion of PTH.[44] The patients also had bilateral, symmetrical, sensorineural deafness involving all frequencies. The renal abnormalities consisted mainly of bilateral cysts that compressed the glomeruli and tubules, and led to renal impairment in some patients. Cytogenetic abnormalities were not detected and abnormalities of the PTH gene were excluded.[44] However, cytogenetic abnormalities involving chromosome 10p14-10pter were identified in 2 unrelated patients with features that were consistent with HDR. These two patients suffered from HDR and mental retardation; one patient also had a solitary dysplastic kidney with vesicoureteric reflux and a uterus bicornis unicollis and the other patient, who had a complex reciprocal, insertional translocation of chromosomes 10p and 8q, had cartilaginous exostoses.[45] Neither of these patients had immunodeficiency or heart defects, which are key features of DGS2 (see earlier), and further studies defined 2 nonoverlapping regions; thus, the DGS2 region was located on 10p13-14 and HDR on 10p14-10pter. Deletion mapping studies in 2 other HDR patients further defined a critical 200 kb region that contained GATA3, which belongs to a family of zinc-finger transcription factors that are involved in vertebrate embryonic development. DNA sequence analysis in other HDR patients identified mutations that resulted in a haploinsufficiency and loss of GATA3 function.[45,46]

GATA3 has two zinc-fingers, and the C-terminal finger (ZnF2) binds DNA, while the N-terminal finger (ZnF1) stabilizes this DNA binding and interacts with other zinc finger proteins, such as the Friends of GATA (FOG).[46] HDR-associated mutations involving GATA3 ZnF2 or the adjacent basic amino acids were found to result in a loss of DNA binding, while those involving ZnF1 either lead to a loss of interaction with FOG2 ZnFs or altered DNA binding affinity (Fig. 34.2).[46] These findings are consistent with the proposed 3-dimensional model of GATA3 ZnF1, which has separate DNA and protein binding surfaces.[46] Thus, the HDR-associated GATA3 mutations can be subdivided into two broad classes, which depend upon whether they disrupt ZnF1 or ZnF2, and their subsequent effects on interactions with FOG2 and altered DNA binding, respectively. The majority (>75%) of these HDR associated mutations are predicted to result in truncated forms of the GATA3 protein. Each proband and family will generally have its own unique mutation and there appears to be no correlation with the underlying genetic defect, and the phenotypic variation, for example, the presence or absence of renal dysplasia. Over 90% of patients with two or three of the major clinical features of the HDR syndrome, that is, hypoparathyroidism, deafness, or renal abnormalities, have a GATA3 mutation.[46] The remaining 10% of HDR of patients who do not have a GATA3 mutation of the coding region, may harbor mutations in the regulatory sequences flanking the GATA3 gene, or else they may represent genetic heterogeneity. The phenotypes of HDR patients with GATA3 mutations appear to be similar to those without GATA3 mutations.[46] It is important to note that HDR patients with GATA3 haploinsufficiency do not have immune deficiency, and this suggests that the immune abnormalities observed in some patients with 10p deletions are most likely to be caused by other genes on 10p. Similarly, the facial dysmorphism, growth, and development delay, commonly seen in patients with larger 10p deletions were absent in the HDR patients with GATA3 mutations, further indicating that these features were likely due to other genes on 10p.[45] These studies of HDR patients indicate an important role for GATA3 in parathyroid development and in the etiology of hypoparathyroidism.

3.2.1 Mouse Model for HDR

The HDR phenotype is consistent with the expression pattern of GATA3 during human and mouse embryogenesis in the developing kidney, otic vesicle, and parathyroids. However, GATA3 is also expressed in the developing central nervous system and the hematopoietic organs in man and mice, and this suggests that GATA3 may have a more complex role. Indeed, studies of *Gata3*$^{+/-}$and *Gata3*$^{-/-}$ mice have revealed important roles for *Gata3* in the development of the brain, spinal cord, peripheral auditory system, T cells, fetal liver hematopoiesis, and urogenital system.[47] *Gata3*$^{-/-}$ mice die between E11.5 and E12.5,[47] but *Gata3*$^{+/-}$ mice are viable, appear to be normal with a normal life span, and are fertile.[47] However, *Gata3*$^{+/-}$ mice have hearing loss and parathyroid abnormalities. The hearing loss is associated with cochlear abnormalities, which consists of a significant progressive morphological degeneration that starts with the outer hair cells at the apex and eventually involves all the inner hair cells, pillar cells, and nerve fibers.[48,49] These studies have shown that hearing loss in *Gata3* haploinsufficient mice commences in the early postnatal period and is progressive through adulthood, and that it is peripheral in origin and is predominantly due to malfunctioning of the outer hair cells of the cochlea.[48,49]

Gata3 loss also resulted in parathyroid abnormalities. Thus, *Gata3*$^{-/-}$ and *Gata3*$^{+/-}$ embryos lacked or had smaller parathyroid-thymus primordia, respectively,[50] and the parathyroids of adult *Gata3*$^{+/-}$ mice did not enlarge or

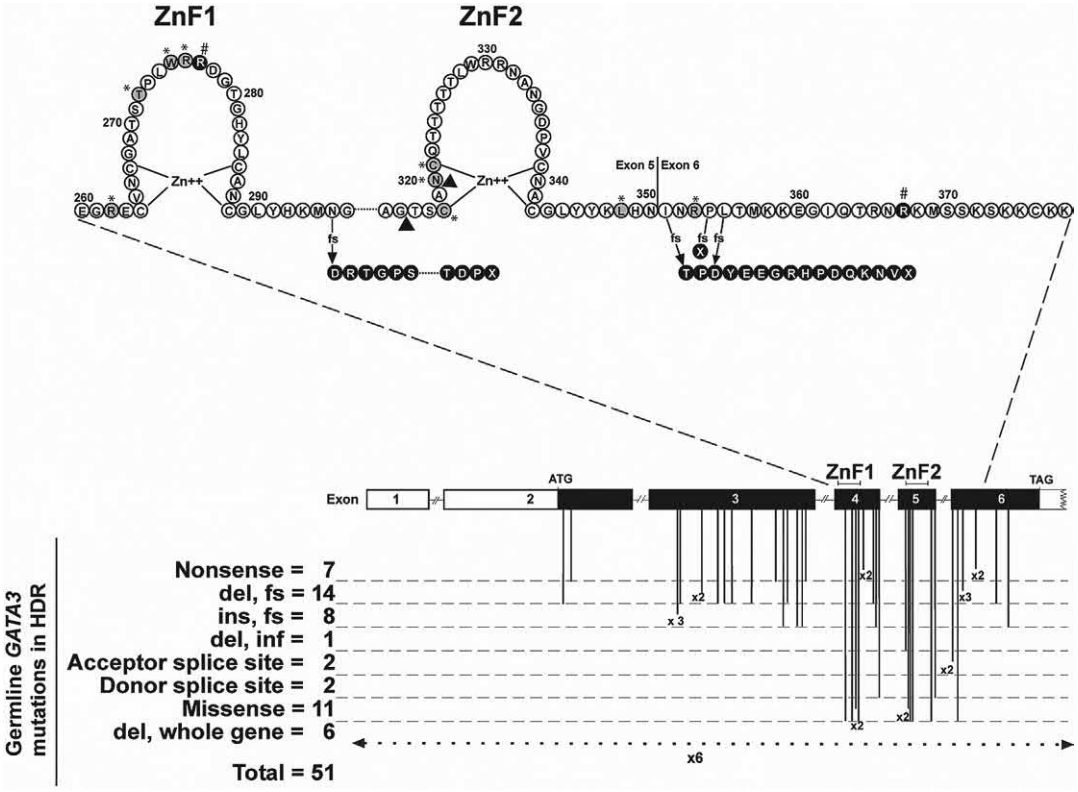

FIGURE 34.2 Schematic representation of the genomic structure of the *GATA3* gene illustrating the locations of mutations identified in Hypoparathyroidism, Deafness and Renal anomalies (HDR) syndrome patients. The human *GATA3* gene consists of 6 exons, spanning 20 kb of genomic DNA, and encodes a 444 amino acid transcription factor that includes 2 zinc fingers (ZnF1 and ZnF2). The ATG (translation start) and TAG (stop) sites are in exons 2 and 6, respectively. The locations of the 51 reported GATA3 mutations associated with HDR are shown. Twenty-five of the 51 HDR mutations, which affect the region encompassing the 2 zinc fingers and the adjacent C-terminal region, are further detailed earlier in the amino acid sequence, in which every tenth amino acid is numbered. The amino acids altered by the 25-HDR mutations are highlighted, with asterisk, representing missense mutations; hash symbol nonsense mutations; black arrowhead, deletion (del); fs, frameshift; inf, inframe. *Source: Adapted from Ali A, Christie PT, Grigorieva IV, et al. Functional characterization of GATA3 mutations causing the hypoparathyroidism-deafness-renal (HDR) dysplasia syndrome: insight into mechanisms of DNA binding by the GATA3 transcription factor.* Hum Mol Genet *2007;***16**:*265–75.*[46]

have an increased proliferation rate in response to hypocalcemia, which was induced by a low calcium/vitamin D diet. Moreover, the adult *Gata3*$^{+/-}$ mice had an inadequate increase in plasma PTH in response to the induced hypocalcemia. These findings in the *Gata3*$^{+/-}$ mice are consistent with the observed hypocalcemia that occurs in association with inappropriately normal or low plasma PTH concentrations in patients who have the HDR syndrome due to *GATA3* haploinsufficiency.[44,45] The smaller size or absence of the parathyroids in the *Gata3*$^{+/-}$ and *Gata3*$^{-/-}$ embryos, respectively, is associated with a markedly reduced number of *Gcm2*-expressing cells in the 3rd pharyngeal pouch and it is likely that GATA3 is critical to maintaining differentiation, and subsequent survival of parathyroid and thymus progenitor cells. Indeed, the gene for glial cells missing B (*GCMB*), which is the human homologue of mouse Gcm2, has been shown to be transcriptionally regulated by GATA3.[50]

Examination of *Gata3*$^{-/-}$ embryos has revealed a variety of abnormalities that included massive internal bleeding, resulting in anemia, marked growth retardation, severe deformities of the brain and spinal cord, a hypopigmented retina, gross aberrations in fetal liver hematopoiesis, a total block of T-cell differentiation, and a retarded or missing lower jaw area.[47,51] These *Gata3*$^{-/-}$ mice had an anatomically normal sympathetic nervous system, yet the sympathetic ganglia lacked tyrosine hydroxylase and dopamine beta-hydroxylase, which are key enzymes that convert tyrosine to L-DOPA, and dopamine to noradrenaline, respectively, in the catecholamine synthesis pathway. Thus, the *Gata3*$^{-/-}$ mice lacked noradrenaline in the sympathetic neurons, and this was contributing to the early embryonic lethality.[51] Feeding of catecholamine intermediates to the pregnant dams, helped to partially rescue the *Gata3*$^{-/-}$ embryos from E12.5 to E16.5.[51] These older, pharmacologically rescued *Gata3*$^{-/-}$ embryos showed abnormalities that could not be detected in the untreated mice.[51] These late embryonic defects included thymic hypoplasia, a thin-walled ventricular septum, a poorly developed mandible, other

developmental defects in structures derived from the cephalic neural crest cells, renal hypoplasia, a failure to form the metanephros, and an aberrant elongation of the nephric duct along the anteroposterior axis of the embryo.[51–53] The defect of the nephric duct, which consisted of an abnormal morphogenesis and guidance in the developing kidney, was characterized by the loss of *Ret* expression that is an essential component of the glial-derived-nerve-factor signaling pathway involved in ureteric bud formation and nephric duct guidance.[53] Thus, *Gata3* has a role in the differentiation of multiple cell lineages during embryogenesis as well as being a key regulator of nephric duct morphogenesis and guidance of the nephric duct in its caudal extension in the pro/mesonephric kidney.[51,53]

3.3 Mitochondrial Disorders Associated With Hypoparathyroidism

Hypoparathyroidism has been reported to occur in three disorders associated with mitochondrial dysfunction: the Kearns–Sayre syndrome (KSS), the MELAS syndrome, and a mitochondrial trifunctional protein (MTP) deficiency syndrome. KSS is characterized by progressive external ophthalmoplegia and pigmentary retinopathy before the age of 20 years, and is often associated with heart block or cardiomyopathy. Symptomatic hypocalcemia due to hypoparathyroidism may be the initial presenting feature of KSS.[54] The MELAS syndrome consists of a childhood onset of mitochondrial encephalopathy, lactic acidosis, and stroke like episodes. In addition, varying degrees of proximal myopathy can be seen in both conditions. Both the KSS and MELAS syndromes have been reported to occur with insulin dependent diabetes mellitus and hypoparathyroidism.[55,56] A point mutation in the mitochondrial gene tRNA leucine (UUR) has been reported in one patient with the MELAS syndrome who also suffered from hypoparathyroidism and diabetes mellitus.[56] Large deletions, consisting of 6741 and 6903 bp and involving more than 38 % of the mitochondrial genome, have been reported in other patients who suffered from KSS, hypoparathyroidism, and sensorineural deafness.[57] Rearrangements and duplication of mitochondrial DNA have also been reported in KSS. MTP deficiency is a disorder of fatty-acid oxidation that is associated with peripheral neuropathy, pigmentary retinopathy, and acute fatty liver degeneration in pregnant women who carry an affected fetus. Hypoparathyroidism has been reported in three cases of MTP deficiency, and presents in the infantile period in association with rhabdomyolysis and polyneuropathy.[58] Hypoparathyroidism caused by MTP deficiency has been associated with homozygous mutations of the *HADHB* gene, which encodes the β-subunit of the MTP protein, in two cases.[58] The role of mutations affecting the mitochondrial genome or *HADHB* gene in the etiology of hypoparathyroidism remains to be further elucidated, and as yet mouse models have not been generated to facilitate in vivo studies.

3.4 Kenny–Caffey, Sanjad–Sakati, and Kirk–Richardson Syndromes

Hypoparathyroidism has been reported to occur in over 50% of patients with the Kenny–Caffey syndrome (KCS) which is associated with short stature, osteosclerosis and cortical thickening of the long bones, delayed closure of the anterior fontanel, basal ganglia calcification, nanophthalmos, and hyperopia.[59] KCS may be inherited as an autosomal recessive (KCS type 1, KCS1) or autosomal dominant (KCS type 2, KCS2) disorder (Table 34.2). Parathyroid tissue could not be found in a detailed postmortem examination of one patient[60] and this suggests that hypoparathyroidism may be due to an embryological defect of parathyroid development. In the Kirk–Richardson (KR) and Sanjad–Sakati (SS) syndromes, which are similar, hypoparathyroidism is associated with severe growth failure and dysmorphic features.[61,62] This has been reported in patients of Middle Eastern origin. Consanguinity was noted in the majority of the families, indicating that this syndrome is inherited as an autosomal recessive disorder. Homozygosity and linkage disequilibrium studies located the KR/SS gene to chromosome 1q42-q43 and molecular genetic investigations have been identified that homozygous mutations of the Tubulin-specific chaperone (TBCE) are associated with the KCS1, and KR/SS syndromes.[63] TBCE encodes one of several chaperone proteins required for the proper folding of α-tubulin subunits and the formation of α-β tubulin heterodimers (Fig. 34.1).[63] Whole exome sequencing has demonstrated KCS2 to be caused by heterozygous missense mutations of the family with sequence similarity 111 member A (*FAM111A*) gene, which encodes a protein with homology to trypsin-like peptidases.[64,65] The FAM111A protein has been revealed to modulate DNA replication and chromatin maturation, and may play a role in embryonic development.[65,66] In contrast to the missense *FAM111A* mutations causing KCS2, a heterozygous *FAM111A* mutation, Ser342del, has been shown to cause a more severe skeletal disorder known as gracile bone dysplasia, which occurs in association with hypoparathyroidism, and represents a perinatally lethal condition.[65]

3.4.1 Mouse Models With Tbce Abnormalities

Mice deleted for Tbce develop a progressive caudiocranial degeneration of their motor axons and die by 6 weeks of age.[67] Mice harboring a missense Tbce mutation (Trp524Gly) have also been reported to have motor neuronpathy, which had a reduced number of

microtubules.[68] Abnormalities of calcium homeostasis have not been reported in these mouse models.

3.5 Additional Familial Syndromes

Single familial syndromes in which hypoparathyroidism is a component have been reported (Table 34.2). The inheritance of the disorder in some instances has been established and molecular genetic analysis of the PTH gene has revealed no abnormalities. Thus, an association of hypoparathyroidism, renal insufficiency and developmental delay has been reported in one Asian family in whom autosomal recessive inheritance of the disorder was established. An analysis of the PTH gene in this family revealed no abnormalities.[69] The occurrence of hypoparathyroidism, nerve deafness, and a steroid-resistant nephrosis leading to renal failure, which has been referred to as the *Barakat syndrome*,[70] has been reported in four brothers from one family, and an association of hypoparathyroidism with congenital lymphedema, nephropathy, mitral valve prolapse, and brachytelephalangy has been observed in two brothers from another family.[71] Molecular genetic studies have not been reported from these two families. Hypoparathyroidism has been reported in a patient who had features of the Dubowitz syndrome, which is an autosomal recessive disorder characterized by intrauterine growth retardation, short stature, microcephaly, mild mental retardation, eczema, and characteristic facies, which included blepharophimosis, ptosis, and micrognathia.[72]

3.6 Pluriglandular Autoimmune Hypoparathyroidism

Hypoparathyroidism may occur in association with autoimmune Addison's disease, candidiasis and two or three of the following: insulin-dependent diabetes mellitus, primary hypogonadism, autoimmune thyroid disease, pernicious anemia, chronic active hepatitis, steatorrhea (malabsorption), alopecia (totalis or areata) and vitiligo. The disorder has also been referred to as either the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) syndrome or the autoimmune polyglandular type 1 syndrome (APS1).[73] This disorder has a high incidence in Finland, and a genetic analysis of Finnish families indicated autosomal recessive inheritance of the disorder. In addition, the disorder has been reported to have a high incidence among Iranian Jews, although the occurrence of candidiasis was less common in this population. Linkage studies of Finnish families mapped the APECED gene to chromosome 21q22.3.[74] Further positional cloning approaches led to the isolation of a novel gene from chromosome 21q22.3. This gene, referred to as AIRE (*a*uto*i*mmune *re*gulator), encodes a 545 amino acid protein that contains motifs characteristic of a transcriptional factor and includes two PHD-type zinc-finger motifs, a proline-rich region and three LXXLL motifs.[75,76] Four AIRE mutations are commonly found in APECED families and these are: Arg257Stop in Finnish, German, Swiss, British, and Northern Italian families; Arg139Stop in Sardinian families; Tyr85Cys in Iranian Jewish families; and a 13 bp deletion in exon 8 in British, Dutch, German, and Finnish families.[76–78] The AIRE protein is mainly localized to the nucleus and mediates E3 ubiquitin ligase activity, which is abolished by missense APECED-causing mutations.[79] AIRE has also been shown to regulate the elimination of organ-specific T cells in the thymus, and thus APECED is likely to be caused by a failure of this specialized mechanism for deleting forbidden T cells, and establishing immunological self-tolerance.[80] Finally, whole genome expression and chromatin immunoprecipitation studies have identified AIRE regulated genes; these genes lack active chromatin markers, such as histone H3, trimethylation (H3/C4Me3), and acetylation (AcH3) on their promoters, but during activation by AIRE, these genes acquire histone H3 modifications that are associated with transcription and RNA polymerase II.[81] Patients with APS1 may also develop other autoimmune disorders in association with organ-specific autoantibodies, which are similar to those in patients with non-APS1 forms of the disease. Examples of such autoantibodies and related diseases are GAD6S autoantibodies in Diabetes Mellitus type 1A and 21-hydroxylase autoantibodies in Addison's disease. Patients with APS1 may also develop autoantibodies that react with specific autoantigens that are not found in non-APS1 patients, and examples of this are autoantibodies to type 1 inferferons, which are present in all APS1 patients,[82] and to NACHT leucine-rich-repeat-protein 5 (NALP5) which is a parathyroid-expressed protein present in 49% of patients with APS1-associated hypoparathyroidism.[83] NALP proteins are essential components of the inflammasome and activate the innate immune system in different inflammatory and autoimmune disorders, such as vitiligo, which involves NALP1, and gout, which involves NALP3.[84] The precise role of NALP5 autoantibodies in APS1-associated hypoparathyroidism remains to be elucidated, and these autoantibodies do not appear to be a sensitive or specific marker for APS1-associated hypoparathyroidism.[85]

3.6.1 Mouse Model With Aire1 Deletion

Aire1$^{-/-}$ mice mimicking the common human 13-bp deletion mutation present with only a mild autoimmune phenotype, with an evident increase in the number of activated T cells and detection of autoantibodies against several organs.[86] At the histological level, lymphocytic infiltration of several organs indicated the development of autoimmunity, although symptoms were mild and

the quality of life for *Aire1*$^{-/-}$ mice appeared equivalent to wild-type littermates, with the exception of male infertility suggesting that additional genetic and/or environmental factors contribute substantially to the overt nature of autoimmunity associated with *Aire1* mutations, even for mutations identical to those found in humans with APECED.[86]

4 ABNORMALITIES OF THE CALCIUM-SENSING RECEPTOR AND G-PROTEIN SUBUNIT α11

The calcium sensing receptor (CaSR), which is a 1078 amino acid G-protein coupled receptor with 7 transmembrane domains and a large 612 amino acid extracellular domain, is predominantly expressed in the parathyroids and kidneys, and is pivotal in extracellular calcium homeostasis by mediating alterations in the release of PTH from the parathyroids in response to changes in extracellular calcium concentrations.[87] Thus, an increase in extracellular calcium leads to CaSR-mediated activation of intracellular signaling components, such as G-protein subunit α11 ($G\alpha_{11}$), which in turn increases the free intracellular calcium concentration and leads to a reduction in transcription of the PTH gene. CaSR mutations that result in a loss-of-function are associated with familial hypocalciuric hypercalcemia.[88] Gain-of-function CaSR abnormalities are associated with three hypocalcemic disorders, which are autosomal dominant hypocalcemia type 1 (ADH1), Bartter syndrome type V (i.e., ADH1 with a Bartter-like syndrome), and a form of autoimmune hypoparathyroidism due to CaSR autoantibodies (Table 34.2).[89] Whereas, gain-of-function mutations of the downstream $G\alpha_{11}$ signaling protein lead to a hypocalcemic disorder known as autosomal dominant hypocalcemia type 2 (ADH2).[90]

4.1 ADH1 and Bartter Syndrome Type V

CaSR missense mutations that result in a gain of function lead to ADH1. In ADH1 patients, the hypocalcemia is usually mild and asymptomatic, but may sometimes be associated with tetany and seizures. Hyperphosphatemia and hypomagnesemia are also notable features and the serum PTH concentrations are in the low-normal range. Because of the insensitivities of previous PTH assays in this range, such patients have often been diagnosed to be hypoparathyroid.[89,91] Treatment with vitamin D or its active metabolites to correct the hypocalcamia in these patients can result in marked hypercalciuria, nephrocalcinosis, nephrolithiasis, and renal impairment.[89,91] Thus, these patients need to be distinguished from those with hypoparathyroidism. One study demonstrated that the urinary calcium-to-creatinine (Ca/Cr) ratio could be used to distinguish ADH1 from hypoparathyroidism, with untreated ADH1 patients having a mean Ca/Cr ratio of >0.3 mmol/mmol, which was similar to the urinary Ca/Cr ratio of unaffected normocalcemic subjects, whereas untreated hypoparathyroid patients had a significantly lower mean Ca/Cr ratio of <0.1 mmol/mmol.[92] However, such differences in the urinary Ca/Cr ratio were not apparent between treated ADH1 and hypoparathyroid subjects,[92] and in most cases, mutational analysis of the *CASR* gene is required for the diagnosis of ADH1. More than 70 different CaSR mutations have been identified to date in individuals affected with ADH.[89] ADH1-causing CaSR mutations are heterozygous missense substitutions in 95% of cases. However, a homozygous CaSR mutation has been reported in an individual with ADH1, and his phenotype was similar to that of heterozygous-affected family members.[93] Structure-function analyses of ADH1-causing CaSR mutations have defined key structural regions of the CaSR that maintain this receptor in an inactive conformation. In particular, gain-of-function mutations cluster within the second peptide loop of the extracellular venus flytrap (VFT) domain (residues 116–136) that is predicted to contribute to the interface of the dimeric CaSR[94] (Fig. 34.3). Mutations affecting this extracellular peptide loop may lead to a gain-of-function by promoting conformational changes, such as dimer rotation that facilitates receptor activation. A second hotspot for ADH-associated mutations is located in a region that encompasses transmembrane domains 6 and 7, and the intervening third extracellular loop of the CaSR (residues 819–837)[95] (Fig. 34.3), and which is likely important for locking the ligand-unbound family C GPCRs in an inactive conformation by forming a network of interactions with other transmembrane domains[96] that inhibits the binding of G-proteins.

Patients with Bartter syndrome type V have the classical features of the syndrome, that is, hypokalemic metabolic alkalosis, hyperreninemia and hyperaldosteronism.[97,98] In addition, they develop hypocalcemia, which may be symptomatic and lead to carpopedal spasm, and an elevated fractional excretion of calcium that may be associated with nephrocalcinosis.[97,98] Such patients have been reported to have heterozygous gain-of-function CaSR mutations, and in vitro functional expression of these mutations has revealed a more severe set-point abnormality for the receptor than that found in patients with ADH1.[97,98] Indeed, some Bartter type V-associated mutations elicited CaSR signal transduction in the absence of stimulation with extracellular calcium, thereby indicating that these mutations were constitutively activating.[98] This suggests that the additional features occurring in Bartter syndrome type V, but not in ADH1, are due to severe gain-of-function mutations of the CaSR.

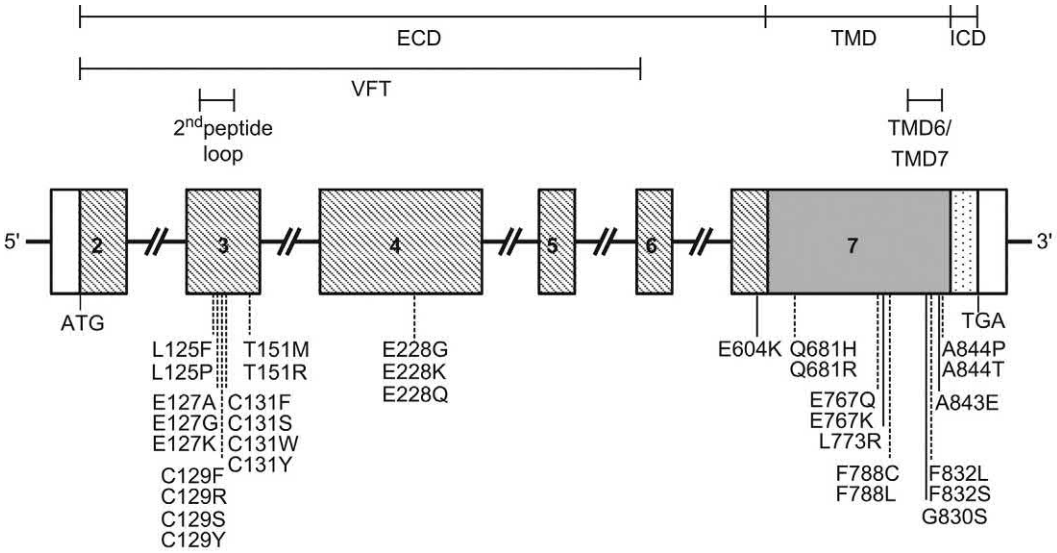

FIGURE 34.3 Schematic representation of the genomic structure of the *CASR* gene illustrating mutational hotspots for ADH1-associated missense mutations. The *CASR* gene consists of 6 coding exons (2–7), and the start (ATG) and stop (TGA) codons are in exons 2 and 7, respectively. The 5′ portion of exon 2, and the 3′ portion of exon 7 are untranslated (open boxes). The 3′ portion of exon 2, exons 3, 4, 5, 6, and the 5′ portion of exon 7, encode the extracellular domain [ECD *(hatched boxes)*], and the midportion of exon 7 encodes the transmembrane domain [TMD *(gray box)*] and intracellular domain [ICD *(dotted box)*]. ADH1-associated missense substitutions cluster at mutational hotspots, which are the site of recurrent missense mutations *(solid lines)* that have been reported in three or more probands (mutational frequency of >1.5%), and/or affected by multiple (3 or more) different missense mutations *(dashed lines)*. These mutational hotspots are located in the 2nd peptide loop of the extracellular venus flytrap (VFT) domain, and in the region encompassing TMD 6 and 7 (TMD6/TMD7).

4.1.1 Mouse Models for ADH1

Mouse models with gain-of-function *Casr* mutations have been generated by chemical mutagenesis and knock-in strategies. One of these mouse models, designated "Nuclear flecks" or "*Nuf*" due to the presence of opaque flecks in the nucleus of the lens, was generated using the alkylating agent isopropyl methane sulphonate.[99] The *Nuf* gene locus was mapped to mouse chromosome 16 and a germline leucine to glutamine missense substitution identified at codon 723 of the *Casr.* The missense CaSR mutation, Leu723Gln, when expressed in HEK293 cells resulted in a significantly lower EC_{50} value, with a gain-of-function.[99] The phenotype of *Nuf* mice consisted of ectopic calcifications, hypocalcemia, hyperphosphatemia, and inappropriately low circulating PTH concentrations,[99] and homozygote (*Nuf/Nuf*) mice had a more severe phenotype than heterozygote (*Nuf/+*) mice.[99] Two strains of *Casr* knock-in mice, which harbor mutations causing ADH1 in humans, have also been generated. These mice had a more marked phenotype than *Nuf* mice, with germline homozygous *Casr* mutations being associated with embryonic or perinatal lethality.[100] Heterozygous-affected *Casr* knock-in mice had a phenotype closely resembling ADH1, and characterized by hypocalcemia, hyperphosphatemia, low plasma PTH, and hypercalciuria.[100]

4.2 ADH2

Mutations of the *CASR* gene are not detected in around 30% of ADH patients, and mutational analysis of $G\alpha_{11}$, which is encoded by the *GNA11* gene, has led to the identification of heterozygous germline $G\alpha_{11}$ mutations in seven ADH patients and families to-date.[90,101–104] In vitro functional studies of these $G\alpha_{11}$ mutations, which all comprise heterozygous missense substitutions, have demonstrated cells expressing the mutant $G\alpha_{11}$ proteins to have enhanced CaSR-mediated signaling responses, consistent with a gain-of-function.[90,101,103] These individuals and families with germline gain-of-function $G\alpha_{11}$ mutations, who were designated as having ADH2, generally had mild-to-moderate hypocalcemia with serum adjusted calcium concentrations ranging from 1.75–2.15 mmol/L, and affected individuals typically presented with hypocalcemic symptoms, such as paresthesia, muscle cramps, carpopedal spasms and seizures.[90,101–104] Moreover, ADH2 has been associated with a milder urinary phenotype, with significantly reduced urinary calcium excretion compared to ADH1 patients,[101] although some ADH2 patients are affected by treatment-related nephrocalcinosis and nephrolithiasis.[101,103] In addition, short stature has been noted in two ADH2 kindreds.[101,104] Structural and functional studies of ADH2-causing gain-of-function $G\alpha_{11}$ mutations have revealed residues and peptide motifs critical for Gα-subunit function. The $G\alpha_{11}$-subunit consists of a Ras-like GTPase domain that binds GDP and GTP, and a smaller helical domain that acts as a clasp to secure these bound guanine-nucleotides. The ADH2-causing mutations have been shown to cluster at the interface between these two domains,[103] and may enhance the exchange of GDP and GTP, thereby

leading to G-protein activation. ADH2 mutations also affect the C-terminal portion of the $G\alpha_{11}$ protein, which facilitates G-protein-GPCR coupling.[103] Thus, these different ADH2-causing mutations have yielded insights into Gα-subunit structure–function relationships, which may aid the design of novel targeted therapeutic agents for the treatment of ADH, for which there is currently a lack of adequate drugs.[105]

4.2.1 Preclinical Studies and Early Clinical Trial of Calcilytics for Treatment of ADH

Calcilytics are compounds that selectively bind to the CaSR and allosterically inhibit the function of this G-protein coupled receptor. There are two main classes of calcilytics, which are the amino alcohols (e.g., NPS-2143, ronacaleret, and JTT-305/MK-5442) and quinazolinones (e.g., ATF936 and AXT914),[106] and these compounds have been demonstrated, in vitro, to improve the gain-of-function associated with ADH-causing CaSR and $G\alpha_{11}$ mutations.[100,107,108] To assess whether calcilytics may ameliorate the hypocalcemia associated with ADH1, these drugs have been administered to mouse models harboring germline gain-of-function CaSR mutations. In a single-dose in vivo study, the NPS-2143 calcilytic compound was administered to *Nuf* mice, which have hypocalcemia and reduced plasma PTH concentrations, in association with a germline gain-of-function CaSR mutation, Leu723Gln.[105] Intraperitoneal injection of NPS-2143 significantly increased plasma calcium and PTH concentrations in heterozygous- and homozygous-affected *Nuf* mice at 1 h after administration, with values returning to baseline after 4 h, and the elevations in plasma calcium induced by NPS-2143 were not associated with any increase in urinary calcium excretion.[105] Longer-term in vivo studies involving the JTT-305/MK-5442 calcilytic compound have been undertaken in two mouse models, which harbor ADH1-causing Cys129Ser and Ala843Glu gain-of-function CaSR mutations, respectively.[100] These studies have demonstrated that administration of JTT-305/MK-5442 by daily oral gavage over a 12 week period led to sustained increases in serum calcium concentrations and a significant reduction in urinary calcium excretion.[100] Moreover, a calcilytic compound known as NPSP795 has been evaluated in a clinical trial involving five ADH patients harboring germline CaSR mutations, and administration of NPSP795 by iv infusion significantly increased plasma PTH concentrations and reduced urinary calcium excretion.[109] However, circulating calcium levels were not altered in this study, and the optimal dosing regimen for NPSP795 remains to be established in ADH patients.

4.3 Autoimmune Acquired Hypoparathyroidism (AH)

Twenty percent of patients who had acquired hypoparathyroidism (AH) in association with autoimmune hypothyroidism, were found to have autoantibodies to the extracellular domain of the CaSR.[110,111] The CaSR autoantibodies did not persist for long; 72% of patients who had AH for less than 5 years had detectable CaSR autoantibodies; whereas only 14% of patients with AH for more than 5 years had such autoantibodies.[111] The majority of the patients who had CaSR autoantibodies were females, a finding that is similar to that found in other autoantibody mediated diseases. Indeed a few AH patients have also had features of APS1. These findings establish that the CaSR is an autoantigen in AH.[110,111] The major epitope for anti-CaSR antibodies has been revealed to be the N-terminal portion of the CaSR (residues 41–69).[112]

5 ISOLATED HYPOPARATHYROIDISM

Isolated hypoparathyroidism may either be *inherited* as an autosomal or X-linked disorder.[5]

5.1 Autosomal Hypoparathyroidism, and PTH Gene Abnormalities

PTH gene abnormalities may lead to autosomal dominant and recessive forms of hypoparathyroidism. Thus, autosomal dominant isolated hypoparathyroidism in 1 patient resulted from a single base substitution (T→C) in exon 2,[113] which led to the substitution of arginine (**C**GT) for cysteine (**T**GT) in the signal peptide. The presence of this charged amino acid in the midst of the hydrophobic core of the signal peptide impeded the processing of the mutant pre-pro-PTH, as demonstrated by in vitro studies. These revealed that the mutation impaired the interaction with the nascent protein and the translocation machinery and that cleavage of the mutant signal sequence by solubilized signal peptidase was ineffective.[113,114] In addition, the mutant PTH has been shown to be trapped intracellularly, predominantly in the endoplasmic reticulum, which is toxic for the cells and leads to apoptosis.[115] PTH mutations have been reported to result in autosomal recessive hypoparathyroidism in two families. In one family, a single base substitution (**T→C**) involving codon 23 of exon 2 was detected. This resulted in the substitution of proline (**C**CG) for the normal serine (**T**CG) in the signal peptide.[116] This mutation alters the −3 position of the pre-pro-PTH protein cleavage site. Indeed, amino acid residues at the −3 and −1 positions of the signal peptidase recognition site have to conform to certain criteria for correct processing through the rough endoplasmic reticulum (RER), and one of these is an absence of proline in the region −3 and +1 of the site. Thus, the presence of a proline, which is a strong helix-breaking residue, at the −3 position is likely to disrupt cleavage of the mutant pre-pro-PTH that would be subsequently degraded in the RER,

and PTH would not be available.[116] Another abnormality of the PTH gene, involving a donor splice site at the exon 2-intron 2 boundary, has been identified in one family with autosomal recessive isolated hypoparathyroidism.[69] This mutation involved a single base transition (**g→c**) at position 1 of intron 2 and an assessment of the effects of this alteration in the invariant **gt** dinucleotide of the 5′ donor splice site consensus on mRNA processing revealed that the mutation resulted in exon skipping, in which exon 2 of the PTH gene was lost and exon 1 was spliced to exon 3. The lack of exon 2 would lead to a loss of the initiation codon (ATG) and the signal peptide sequence, which are required respectively for the commencement of PTH mRNA translation and for the translocation of the PTH peptide. Recently, a homozygous arginine-to-cysteine mutation was identified at codon 25 of the mature PTH (1–84) peptide in a family with hypocalcemia and hyperphosphatemia.[117] The plasma PTH levels of affected family members varied from low-normal to markedly elevated, depending on the type of PTH assay used.[117] Administration of recombinant PTH (1–34) increased urinary cAMP excretion in affected family members, thus indicating they did not have PHP.[117] In contrast to previously reported PTH gene mutations, which affect secretion of this hormone, the Arg25Cys missense substitution was shown to diminish the binding of the mutant PTH peptide with the PTH1 receptor.[117] Moreover, the Arg-25Cys mutation interfered with PTH immunoassays that utilized antibodies affinity-purified using PTH 1–34 and 13–34 fragments, thus explaining why some assays were unable to detect the mutant PTH peptide.[117]

5.1.1 *Mouse Model With Pth deletion*

Mice deleted for the Pth gene have been generated. $Pth^{+/-}$ mice were viable with no apparent phenotypic abnormalities. However, PTH null ($Pth^{-/-}$) mice had enlarged parathyroid glands with an absence of PTH expression, but with substantial CaSR expression. $Pth^{-/-}$ mice also had abnormal skull formation with enhanced mineralization, along with shortening of the long bones and other bone abnormalities.[118] When maintained on a normal calcium diet, $Pth^{-/-}$ mice developed hypocalcemia and hyperphosphatemia, consistent with hypoparathyroidism, that was associated with an increased serum $1,25(OH)_2D_3$ concentration. Moreover, when given a low calcium diet, serum $1,25(OH)_2D_3$ concentrations further increased in the $Pth^{-/-}$ mice, and this led to increased bone resorption and a maintenance of serum calcium at the expense of osteopenia.[119]

5.2 Autosomal Hypoparathyroidism and GCMB Abnormalities

Studies of patients with isolated hypoparathyroidism have shown that *GCMB* mutations are associated with autosomal recessive and dominant forms of the disease.[120–124] Thus, a homozygous intragenic deletion of *GCMB* has been identified in a patient with autosomal recessive hypoparathyroidism,[122] while in other families a homozygous mutation (Arg47Leu) of the DNA binding domain has been reported.[120] Four different homozygous germline mutations have been identified in eight families that originate from the Indian Subcontinent [Arg39Stop, Arg47Leu, Arg110Trp, and a frameshifting deletion (I298fsX307)].[121] Functional analysis using subcellular localization studies, electrophoretic mobility shift assays and luciferase-reporter assays demonstrated that: the Arg39Stop mutant failed to localize to the nucleus; the Arg47Leu and Arg110Trp mutants both lost DNA-binding ability; and the I298fsStop307 mutant had reduced transactivational ability.[121] More recently, three heterozygous *GCMB* mutations, which consist of single nucleotide deletions (c1389deT and c1399delC) that introduce frameshifts and premature truncations, have been identified in two unrelated families with autosomal dominant hypoparathyroidism,[123] and a missense mutation Asn502His was identified in one family with autosomal dominant hypoparathyroidism.[124] These three mutations were shown, by using a GCMB-associated luciferase reporter assay, to inhibit the action of the wild-type transcription factor, thereby indicating that these GCMB mutants have dominant-negative properties.[123,124]

5.2.1 *Mouse Model With Gcm2 Deletion*

$Gcm2^{-/-}$ mice lack parathyroid glands and develop hypocalcemia and hyperphosphatemia as observed in hypoparathyroidism.[125,126] However, despite their lack of parathyroid glands, $Gcm2^{-/-}$ mice do not have undetectable serum PTH levels.[125,126] This endogenous level of PTH in the $Gcm2^{-/-}$ mice is too low to correct the hypocalcemia, but exogenous continuous PTH infusion could correct the hypocalcemia.[125] Interestingly, there were no compensatory increases in PTHrP or $1,25(OH)_2D_3$. These findings indicate that $Gcm2^{-/-}$ mice have a normal response (and not resistance) to PTH. Long-term treatment of the $Gcm2^{-/-}$ mice with $1,25(OH)_2D_3$ restored the serum calcium concentrations to normal and reduced the serum PTH levels, thereby indicating that the production of PTH can be downregulated.[125] This Gcm2-independent auxiliary source of PTH production has been shown to be from the medullary thymic epithelial cells, in which PTH is expressed as a self-antigen for negative selection.[126]

The specific role of *Gcm2* in the development of the parathyroids from the 3rd pharyngeal pouch has been further investigated by studying the expression of the Hoxa3-Pax1/9-Eya1 transcription factor and sonic hedgehog–bone morphogenetic protein 4 (Shh–Bmp4) signaling networks.[43] These studies have revealed that *Gcm2* expression begins at E9.5 in the dorsal anterior pharyngeal endoderm of the 3rd pouch and is maintained in

the presumptive mouse parathyroid domain at later stages,[127] and that at E12.0 *Gcm2*$^{-/-}$ embryos have a parathyroid-specific domain, but that this parathyroid domain undergoes coordinated programmed cell death (apoptosis) by E12.5 in the *Gcm2*$^{-/-}$ mouse embryos.[43] Moreover, the expression of the transcription factors Hoxa3, Pax1, Pax9, Eya1, and Tbx1, and of Shh and Bmp4 was normal in the 3rd pharyngeal pouches of these *Gcm2*$^{-/-}$ mouse embryos. These findings indicate that the Hoxa3-Pax1/9-Eya transcription factor cascade, the transcription factor Tbx1 and the Shh-Bmp4 signaling network, all act upstream of Gcm2.[43] Indeed it has been shown that *Hoxa3* is required for the initiation of *Gcm2* expression in the 3rd pouch endoderm, and both *Hoxa3* and *Pax1* are required for the maintenance of *Gcm2* expression.[128] Moreover, these studies have revealed that Gcm2 has a role in promoting differentiation and survival of parathyroid cells in the developing embryo.[43] Thus, *Gcm2* is required for the differentiation of parathyroid precursor cells in the parathyroid specific domain, but is not required for initial patterning or expression of differentiation markers, such as the CaSR in the common parathyroid/thymus primordia.[43] The target genes of mammalian GCMB/Gcm2 are largely unknown. However, studies that utilized cultured primary parathyroid cells from hyperplastic glands of patients with chronic kidney disease[129] have demonstrated that downregulation of GCMB expression achieved by infection with lentivirus expressing shRNA for GCMB, resulted in downregulation of CaSR expression, thereby suggesting that one of the functions of GCMB may be to maintain high levels of CaSR expression in parathyroid cells.[129] These findings are supported by studies in cotransfected HEK-293, in which exogenous GCMB was able to transactivate reporter constructs that contained CaSR promoter DNA sequences, which encompassed GCMB response elements.[130]

5.3 X-linked Recessive Hypoparathyroidism

X-linked recessive hypoparathyroidism has been reported in two multigenerational kindreds from Missouri, USA.[131,132] In this disorder only males are affected and they suffer from infantile onset of epilepsy and hypocalcemia, which is due to an isolated defect in parathyroid gland development.[133,134] Relatedness of the two kindreds has been established by demonstrating an identical mitochondrial DNA sequence, which is inherited via the maternal lineage, in affected males from the two families.[135] Studies utilizing X-linked polymorphic markers in these families localized the mutant gene to chromosome Xq26-q27, and a molecular deletion–insertion that involves chromosome 2p25 and Xq27 has been identified.[134,136] This deletion-insertion is located approximately 67 kb downstream of SOX3, and hence it is likely to exert a position effect on SOX3 expression. Moreover, SOX3 was shown to be expressed in the developing parathyroids of mouse embryos, and this indicates a likely role for SOX3 in the embryonic development of the parathyroid glands.[136] SOX3 belongs to a family of genes encoding high-mobility group box transcription factors and is related to SRY, the sex-determining gene on the Y chromosome. The mouse homologue is expressed in the prestreak embryo and subsequently in the developing CNS, which includes the region of the ventral diencephalons involved in the development of the anterior pituitary, and which gives rise to the hypothalamus, olfactory placodes, and parathyroids.[136–139] The location of the deletion-insertion ~67 kb downstream of SOX3 in X-linked recessive hypoparathyroid patients is likely to result in altered SOX3 expression, as SOX3 expression has been reported to be sensitive to position-effects caused by X-chromosome abnormalities.[140] Indeed, reporter-construct studies of the mouse Sox3 gene have demonstrated the presence of both 5′ and 3′ regulatory elements,[141] and thus it is possible that the deletion-insertion in the X-linked recessive hypoparathyroid patients may have a position-effect on SOX3 expression, and parathyroid development from the pharyngeal pouches. Indeed such position-effects on SOX genes, which may be exerted over large distances, have been reported. For example, the very closely related Sox2 gene has been shown to have regulatory regions spread over a long distance, both 5′ and 3′ to the coding region[142] and disruption of sequences at some distance 3′ have been reported to lead to loss of expression in the developing inner ear, and absence of sensory cells, whereas expression in other sites is unaffected.[143] Similarly for the SRY gene, which probably originated from SOX3,[144] both 5′ and 3′ deletions result in abnormalities of sexual development, and translocation breakpoints over 1 Mb upstream of the SOX9 gene have been reported to result in Campomelic dysplasia due to removal of elements that regulate SOX9 expression.[140] The molecular deletion-insertion identified in X-linked recessive hypoparathyroidism may similarly cause position-effects on SOX3 expression, and this points to a potential role for the SOX3 gene in the embryological development of the parathyroid glands from the pharyngeal pouches.

6 GENE TESTING IN CLINICAL PRACTICE

Familial hypoparathyroidism may present at any time ranging from the neonatal period until well into adulthood, and occur in isolation or in association or as part of congenital syndromes, and have an autosomal dominant, autosomal recessive, or X-linked recessive mode of inheritance. Chromosome 22q11.2 deletions account for up to 60% of hypoparathyroidism cases

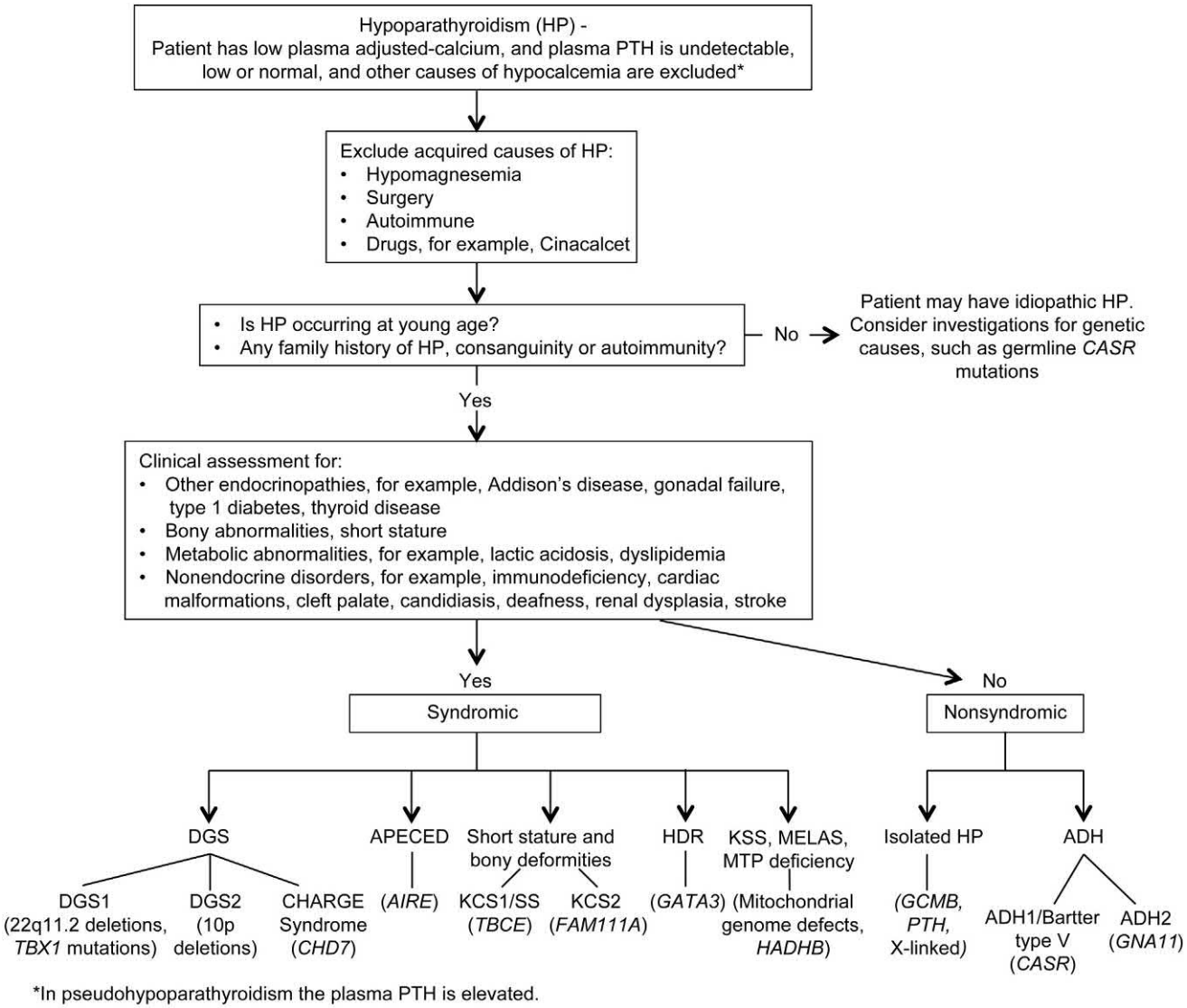

FIGURE 34.4 Clinical approach to establishing the genetic etiology of hypoparathyroidism. The genes for each disorder are indicated in italics, and further details are provided in Table 34.2. APECED, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy; KCS1, Kenny–Caffey syndrome type 1; KCS2, Kenny–Caffey syndrome type 2; SS, Sanjad–Sakati syndrome; HDR, Hypoparathyroidism, deafness and renal anomalies; KSS, Kearns–Sayre syndrome; MELAS, mitochondrial encephalopathy, lactic acidosis and stroke like episodes; MTP, mitochondrial trifunctional protein. *Source: Adapted from Thakker RV, Bringhurst FR, Juppner H. Regulation of calcium homeostasis and genetic disorders that affect calcium metabolism. In: De Groot LJ, Jameson JL, editors.* Endocrinology. *7th ed. Philadelphia, PA: Elsevier; 2016:1063–89.*[5]

that have a childhood age of onset,[13] while single gene mutations, particularly affecting *GATA3*, *GCM2*, *CASR*, *AIRE*, *FAM111A*, and the mitochondrial genes, have been reported to be present in up to 20% of childhood-onset cases.[13,145] In contrast, in patients with adult-onset idiopathic hypoparathyroidism, genetic abnormalities account for <15% of cases, with *CASR* mutations, which may occur de novo, being most commonly identified in this patient group.[146] An assessment for chromosomal abnormalities or single gene mutations should be considered for hypoparathyroid patients with a high suspicion of a genetic etiology,for example, young age of onset, or family history of hypoparathyroidism or consanguinity. A clinical approach to genetic testing in a patient with hypoparathyroidism is outlined in Fig. 34.4. Genetic testing is helpful in clinical practice for the following reasons: (1) to establish the diagnosis so that screening for associated endocrinopathies or organ dysfunction can be undertaken; (2) individualization of treatment, for example, avoiding active vitamin D preparations in ADH, which may lead to hypercalciuric renal complications; (3) identification of asymptomatic family members who harbor the mutation, and thus require screening for the development of hypoparathyroidism and associated endocrinopathies or other disorders; and (4) to reassure relatives who have not

TABLE 34.3 Pharmaceutical Preparations of Vitamin D and Active Metabolites

Drug	Calciferol (vitamin D_3 or D_2)[a]	Dihydrotachysterol (DHT)[b]	Calcifediol (25-hydroxyvitamin D_3)	Calcitriol ($1,25(OH)_2D_3$)	Alfacalcidol ($1\alpha(OH)D_3$)
	Capsules, 0.25 mg and 1.25 mg	Liquid, 0.25 mg/mL	Capsules, 20 and 50 µg	Capsules, 0.25 and 0.5 µg	Capsules, 0.25, 0.50, and 1 µg
				Injection, 1 µg/mL	Liquid, 2 µg/mL Injection, 2 µg/mL in propylene glycol
Time to maximum effect	4–10 weeks	2–4 weeks	4–20 weeks	0.5–1 week	0.5–1 week
Persistence of effect after cessation	6–30 weeks	2–8 weeks	4–12 weeks	0.5–1 week	0.5–1 week

[a]*Calciferol may contain cholecalciferol or ergocalciferol.*
[b]*Dihydrotachysterol not available in many countries.*
Reproduced from Thakker RV. Hypocalcaemic disorders, hypoparathyroidism, and pseudohypoparathyroidism. In: Wass JAH and Stewart PF, editors. Oxford Textbook of Endocrinology and Metabolism, *2nd ed. Oxford: Oxford University Press; 2011. p. 675–86.*[7]

inherited the causative mutation, that further follow-up is not required.[1]

7 TREATMENT

The three major groups of drugs available for the treatment of hypocalcemia are: supplemental calcium, about 10–20 mmol calcium 6–12 h; vitamin D preparations;[147–149] and recombinant human PTH (rPTH). Patients with hypoparathyroidism seldom require calcium supplements after the early stages of stabilization on vitamin D. A variety of vitamin D preparations have been used (Table 34.3). These include: vitamin D3 (cholecalciferol) or vitamin D2 (ergocalciferol), 25,000–100,000 units (1.25–5 mg/day); dihydrotachysterol (now seldom used), 0.25–1.25 mg/day; alfacalcidol (1α-hydroxycholecalciferol), 0.25–1.0 µg/day; and calcitriol (1,25-dihydroxycholecalciferol), 0.25–2.0 µg/day. In children, these preparations are prescribed in doses based on body weight. Cholecalciferol and ergocalciferol are the least expensive preparations, but have the longest durations of action and may result in prolonged toxicity. The other preparations, which do not require renal 1α-hydroxylation, have the advantage of shorter half-lives and thereby minimize the risk of prolonged toxicity. Calcitriol is probably the drug of choice because it is the active metabolite and, unlike alfacalcidol, does not require hepatic 25-hydroxylation. Close monitoring (at about 1–2 week intervals) of the patient's serum and urine calcium are required initially, and at 3–6 monthly intervals once stabilization is achieved. The aim is to avoid hypercalcemia, hypercalciuria, nephrolithiasis, and renal failure. It should be noted that hypercalciuria may occur in the absence of hypercalcemia. Some patients may present with symptoms of acute hypocalcemia. The management of acute hypocalcemia depends on the severity of the hypocalcemia, the rapidity with which it developed and the degree of neuromuscular irritability. Treatment should be given to symptomatic patients (e.g., with tetany). The preferred treatment for acute symptomatic hypocalcemia is intravenous calcium gluconate.[149]

Although, the use of calcium and active vitamin D preparations, such as calcitriol, can restore normocalcemia in hypoparathyroid patients, these therapies do not rectify the increased urinary calcium excretion, which occurs in the absence of PTH secretion. Indeed, hypercalciuria can occur in calcitriol-treated patients despite serum calcium being maintained at low-normal or below-normal concentrations.[150] PTH replacement therapy has the potential to normalize serum calcium while reducing the risk of hypercalciuria. In support of this, subcutaneous administration of the recombinant biologically active N-terminal 1–34 amino acid fragment of PTH [PTH (1–34)] has been demonstrated to increase serum calcium in hypoparathyroid patients, while leading to a significant reduction in urinary calcium excretion compared to affected individuals treated with calcitriol.[150] However, due to the short half-life of rPTH (1–34), this recombinant peptide is administered by multiple daily injections or as a continuous infusion to maintain normocalcemia over a 24-h period.[151] In contrast, the rPTH (1–84) molecule has a longer half-life, and once daily subcutaneous injections have been shown to maintain serum calcium concentrations within the normal range in hypoparathyroid patients over a 24-h period.[152] Moreover, a phase 3 randomized controlled clinical trial has demonstrated that once daily administration of rPTH (1–84) was effective at reducing the requirements for calcium and active vitamin D preparations in hypoparathyroid patients while maintaining stable serum calcium values.[153] In addition to its effects on mineral metabolism, rPTH (1–84) therapy has been shown to significantly improve quality of life outcomes for hypoparathyroid patients,[11] and this recombinant form of PTH is now licensed in the US as

an adjunct to calcium and vitamin D for the long-term management of hypoparathyroidism. However, treatment with PTH may not prevent patients with ADH from developing hypercalciuric renal complications,[154] and further evaluation of calcilytic compounds, which represent a potential targeted therapy for this disorder, is awaited.

8 CONCLUSIONS

Studies of patients with hypoparathyroidism and mice with parathyroid defects have elucidated important roles for: transcription factors (e.g., TBX1, GATA3, GCMB, AIRE1, and SOX3), the TBCE, and the mitochondrial genome in determining the development of parathyroid glands from pharyngeal pouch cells; the CaSR and $G\alpha_{11}$ in regulating extracellular calcium and the secretion of PTH; and *PTH* gene expression for the synthesis, secretion and action of PTH. Furthermore, clinical studies have shown recombinant PTH to be effective for the management of hypoparathyroidism, and calcilytic compounds have been demonstrated as a potential targeted therapy for ADH.

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CHAPTER

35

$G_s\alpha$, Pseudohypoparathyroidism, Fibrous Dysplasia, and McCune–Albright Syndrome

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1 INTRODUCTION

Heterotrimeric guanine nucleotide-binding proteins (G proteins) couple a superfamily of heptahelical, cell surface receptors to various effector enzymes and ion channels. Agonist binding to G protein-coupled receptors modulates intracellular second messengers and thereby regulates acute and longer-term changes in cell physiology. G_s is a ubiquitously expressed G protein that couples receptors for numerous hormones and neurotransmitters to stimulation of cAMP formation by adenylyl cyclase (Fig. 35.1). Like all G proteins, G_s is composed of an α subunit ($G_s\alpha$) associated with βγ complexes that undergoes activation via a GTPase cycle in which interaction of the inactive G_s heterotrimer (GDP-bound $G_s\alpha/\beta\gamma$) with ligand-bound receptor leads to dissociation of bound GDP and binding of GTP to $G_s\alpha$ (Fig. 35.1). GTP-bound $G_s\alpha$ dissociates from βγ and activates adenylyl cyclase. Inactivation occurs by an intrinsic GTPase activity in $G_s\alpha$ that hydrolyzes bound GTP to GDP, allowing reassociation with βγ.

Germline mutations that inactivate $G_s\alpha$ impair agonist stimulation of cAMP formation in all cells. $G_s\alpha$ is encoded by the complex imprinted gene locus *GNAS* on chromosome 20.[1] Homozygous loss of function mutations are presumed to be lethal, but heterozygous mutations or *GNAS* imprinting defects lead to $G_s\alpha$ deficiency and a variety of clinical disorders involving both defects in hormone action and skeletal development. In contrast, $G_s\alpha$ gain-of-function mutations that impair the GTPase turn-off mechanism, even when heterozygous, are presumed to be germline lethal. Somatic gain-of-function mutations lead to constitutive cAMP formation. Phenotypic manifestations vary widely, depending on the developmental timing of somatic mutation and resultant extent of tissue distribution.

In the following sections, we describe in detail the molecular genetic basis and clinical implications of diseases caused by *GNAS* mutations, including pseudohypoparathyroidism, fibrous dysplasia, and McCune–Albright syndrome.

2 PSEUDOHYPOPARATHYROIDISM/ ALBRIGHT HEREDITARY OSTEODYSTROPHY

2.1 Brief Clinical Description

Pseudohypoparathyroidism (PHP) is defined as resistance to the actions of PTH, primarily in the renal proximal tubule, where PTH acts to stimulate the production of 1,25 dihydroxyvitamin D ($1,25(OH)_2D$) and to prevent reabsorption of phosphate leading to increased urinary phosphate excretion. The biochemical features of PHP are hypocalcemia and hyperphosphatemia with elevated serum PTH levels in the absence of renal failure or 25-hydroxyvitamin D deficiency. Forms of PHP associated with $G_s\alpha$ defects and resulting in impaired stimulation of cAMP production by PTH include PHP types 1A and 1B (PHP1A and PHP1B, respectively). In addition to renal PTH resistance, PHP1A patients also present

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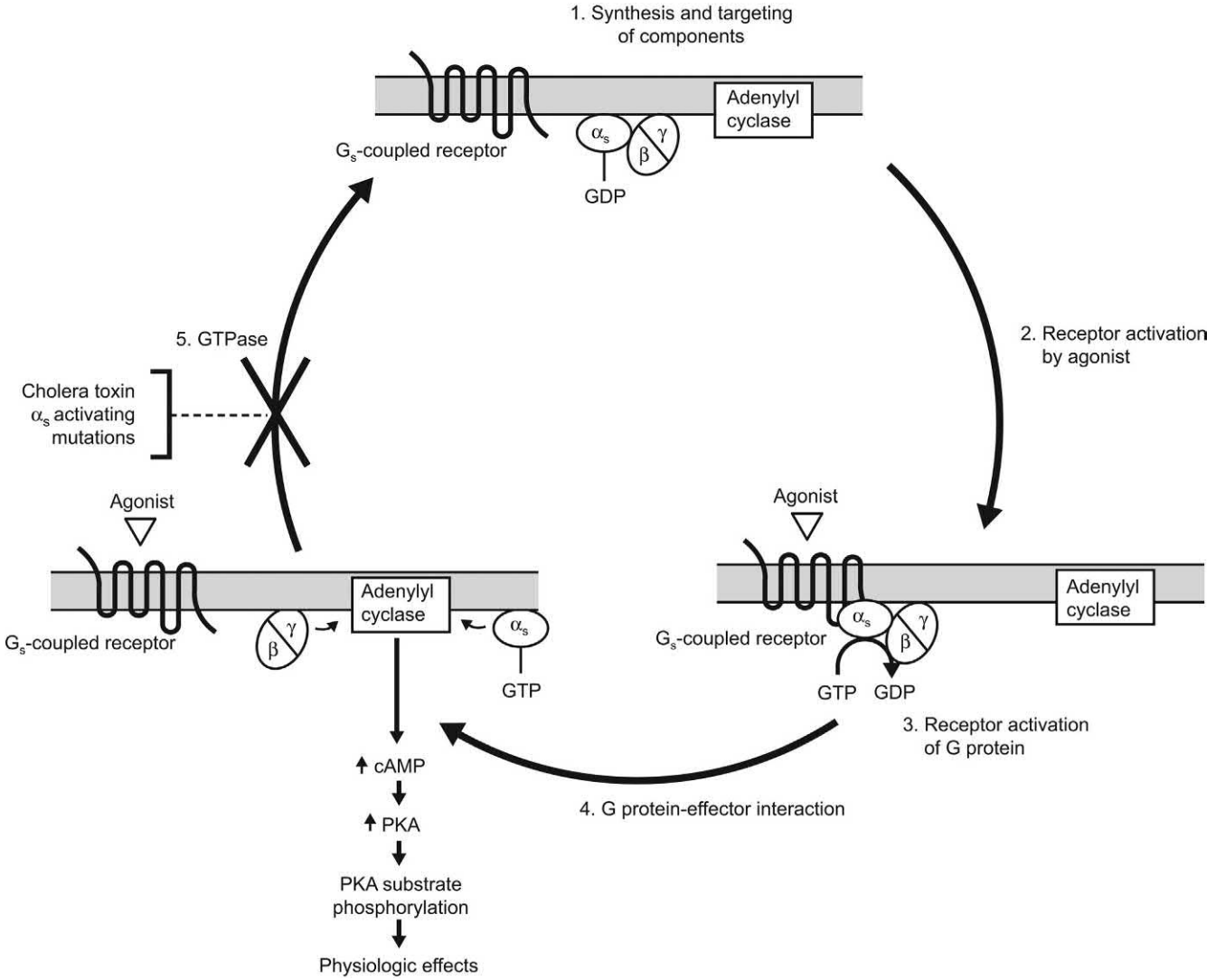

FIGURE 35.1 The G protein GTPase cycle. Potential sites for disease-causing abnormalities are numbered. In each panel, the stippled region denotes the plasma membrane with extracellular (above) and intracellular (below). Under physiologic conditions, effector regulation by G protein subunits is transient and is terminated by the GTPase activity of the α subunit. The latter converts bound GTP to GDP, thus returning the α subunit to its inactivated state with high affinity for the βγ dimer, which reassociates again to form the heterotrimer. The G protein shown is G_s with its effector, adenylyl cyclase. Activation of adenylyl cyclase generates the intracellular 2nd messenger, cAMP, which activates protein kinase A (PKA). The latter enzyme phosphorylates a variety of proteins that mediate the physiologic effects of agonists for G_s-coupled receptors. Cholera toxin covalently modifies the $G_s\alpha$ subunit at residue R201, blocking its GTPase activity. Somatic mutations of the $G_s\alpha$ subunit at either R201 or Q227 likewise block GTPase activity. In both cases, constitutive activation and agonist-independent cAMP formation result.

with obesity, multihormone resistance, and Albright hereditary osteodystrophy (AHO), a congenital syndrome with one or more of the following clinical features: short stature, brachydactyly, subcutaneous ossifications, centripetal facial abnormalities, including depressed nasal bridge or hypertelorism, and mental deficits or developmental delay.[1] (Fig. 35.2) In contrast, PHP1B patients generally do not present with the AHO phenotype or resistance to other hormones (except for borderline TSH resistance). PHP1C is a classification given for rare patients who clinically mimic PHP1A, but for whom no $G_s\alpha$ defect has been identified.

PHP1A and PHP1B patients typically present with hypocalcemic symptoms in mid- to late-childhood, although biochemical evidence of elevated PTH or hyperphosphatemia develops at an earlier age, usually in the first 1–2 years of life. Typically $1,25(OH)_2D$ levels are low or low-normal. Some patients may never develop hypocalcemic symptoms or may even remain eucalcemic. Patients often develop features seen in primary hypoparathyroidism, such as basal ganglia calcifications and cataracts, and rarely will develop rachitic bone changes. While one study showed bone mineral density to be maintained in PHP1A,[2] PHP1B patients may present

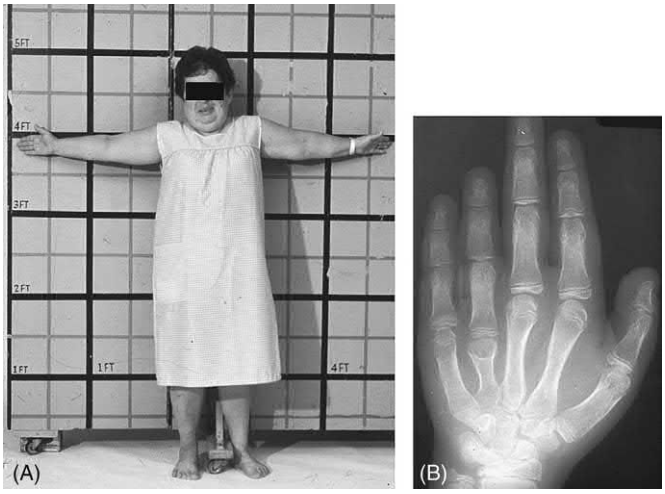

FIGURE 35.2 Albright hereditary osteodystrophy. (A) An AHO patient with short stature, obesity, and rounded face and (B) radiograph of affected hand with brachydactyly affecting the fourth metacarpal.

with low bone density or skeletal features reminiscent of hyperparathyroidism, such as periosteal resorption or even osteitis fibrosa cystica.[3]

PHP1A patients also present with resistance to multiple hormones that also activate $G_s\alpha$ in their target tissues, including thyrotropin (TSH), gonadotropins, and growth hormone-releasing hormone (GHRH). TSH resistance is often detected at birth and is associated with mild to moderate nongoitrous hypothyroidism. Gonadotropin resistance presents primarily in female patients with delayed or incomplete sexual maturation, oligomenorrhea, and/or infertility.[4] Although estrogen levels are often low, circulating gonadotropins are not markedly elevated. Many PHP1A patients are growth hormone deficient due to GHRH resistance, although this may not be the major cause for short stature as these patients also have primary defects in the growth plate leading to premature closure.[5] Prolactin deficiency and impaired olfaction have also been reported in PHP1A patients. Early-onset obesity and primary insulin resistance (not accounted for by obesity) is also a feature of PHP1A.[6,7] It should be noted that in PHP1A, there is no clinical resistance to other hormones that activate $G_s\alpha$, such as vasopressin, glucagon, and ACTH. Multihormone resistance and obesity are generally absent in PHP1B, except for the presence of borderline TSH resistance in about half of the patients.[8–10] Growth hormone deficiency has also been rarely reported in PHP1B.[11,12]

The extent and severity of AHO features in PHP1A patients is quite variable. The most specific feature is ectopic ossifications (osteoma cutis), which are generally limited to the dermis and subcutaneous tissues, and present as palpable hard nodules or as calcifications on radiographs. Rarely, the lesions coalesce to form plate- or cast-like structures and invade into deep tissue, such as muscle, leading to joint stiffness and bone deformity, which is referred to as progressive osseous heteroplasia (POH).[13]

Brachydactyly manifests as shortening and widening of long bones in the hands and feet, most often the distal thumb and third, fourth, and fifth metacarpals, which is due to premature closure of the growth plate associated with coning of the epiphysis.[14] Often the involvement is assymetric. Other musculoskeletal features that have been reported in AHO include spinal cord compression, carpal tunnel syndrome,[15] increased incidence of sleep apnea,[16] dental abnormalities,[17] and more rarely Madelung deformity (subluxation of ulnar head at the wrist)[18,19] and acroscyphodysplasia.[20] Another feature of AHO includes neurocognitive abnormalities, including developmental delay and mental retardation. AHO is associated with low birth weight while PHP1B is associated with high birth weight.[21,22] Some features of AHO, particularly mild brachydactyly, can be present in a minority of patients with the molecular genetic diagnosis of PHP1B.

2.2 Genetics Description

AHO is an autosomal dominant disorder affected by genomic imprinting in which inactivating $G_s\alpha$ mutations inherited from the father (or de novo mutation on the paternal allele) result in only the AHO phenotype (also known as pseudopseudohypoparathyroidism, PPHP); whereas those inherited from the mother (or de novo mutations on the maternal allele) result in AHO plus early-onset obesity and multihormone resistance (PHP1A). A recent report suggests that the neurocognitive abnormalities in AHO may be more prominent in PHP1A than in PPHP patients.[23]

POH is associated with the same mutations as AHO and may present with or without other features of AHO or PHP1A. There may be a predilection for patients who present with POH alone to inherit their mutations from the father, although even within these families, female POH patients may have affected offspring with classic AHO.[24] POH is not associated with the *GNAS* epigenetic changes that are the hallmark of PHP1B.[25] It has been suggested that POH may be a type 2 segmental manifestation caused by somatic loss or mutation of the nonmutated allele during an early developmental stage.[26]

PHP1B (PTH resistance without AHO) can occur either sporadically or in a familial setting in an autosomal dominant pattern (AD-PHP1B). In AD-PHP1B, maternal transmission leads to PTH resistance, whereas paternal transmission leads to a silent carrier state. Only offspring of the female silent carriers present with PTH resistance.[27]

2.3 Molecular Genetics

The $G_s\alpha$ gene *GNAS* at 20q13 is a complex locus that generates multiple gene products via the use of

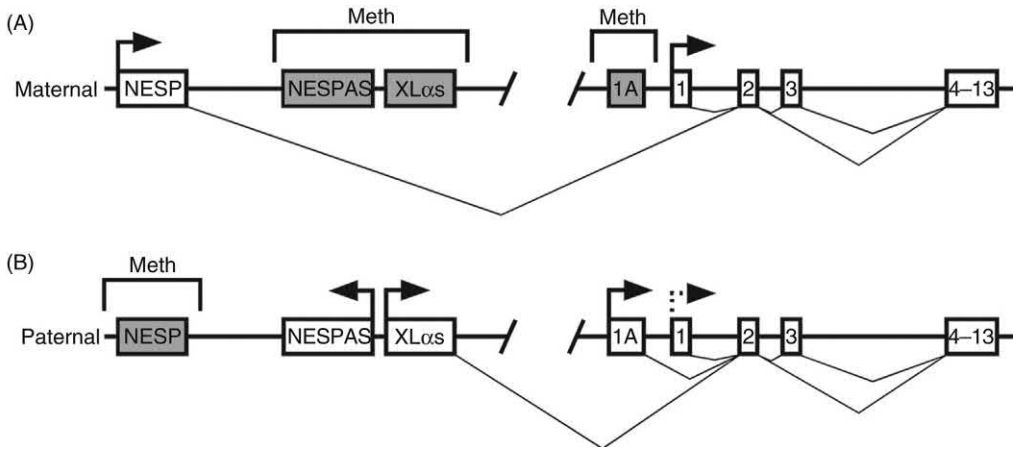

FIGURE 35.3 General organization of the imprinted *GNAS* locus. (A) The *GNAS* maternal and (B) paternal alleles are shown (not to scale) with DNA methylated regions (Meth) and splicing patterns below each allele. The promoters and first exons for NESP55 (NESP), XLαs, exon 1A transcripts, and $G_s\alpha$ (exon 1), as well as the antisense transcripts (NESPAS), are shown with active promoters indicated with an *arrow* and inactive promoters indicated by gray shading. The *striped arrow* for the paternal $G_s\alpha$ promoter indicates that the promoter is silenced in some tissues. $G_s\alpha$ exons 4–13 are shown as a single box. Long and short forms of $G_s\alpha$ result from alternative splicing of exon 3.

alternative first exons that splice onto a common set of downstream exons 2–13 (Fig. 35.3). In addition, the different gene products have differing patterns of allele-specific expression due to genomic imprinting. $G_s\alpha$ is generated from the most downstream promoter region (exon 1) and is biallelically expressed in most tissues, but is expressed primarily from the maternal allele in some tissues, including renal proximal tubules, thyroid, gonad, pituitary somatotrophs, and certain brain regions.[1,28] Just upstream of the $G_s\alpha$ promoter is a differentially methylated region (DMR) that includes a promoter and alternative first exon 1A (also referred to as A/B) that generates noncoding mRNAs of unclear significance. This DMR is critical for the establishment of tissue-specific $G_s\alpha$ imprinting (see below). Long and short forms of $G_s\alpha$ are generated by splicing in and out of exon 3, respectively.

Further upstream are two oppositely imprinted DMRs containing promoters that generate transcripts for the $G_s\alpha$ isoform XLαs and the neuroendocrine-specific protein NESP55, respectively (Fig. 35.3).[1,28] NESP55 is only expressed from the maternal allele and its promoter is DNA methylated on the paternal allele; whereas XLαs is only expressed from the paternal allele and its promoter is methylated on the maternal allele. NESP55 is a chromogranin-like protein unrelated to $G_s\alpha$ and of unclear biological significance while XLαs is a relatively neuroendocrine-specific $G_s\alpha$ isoform that is capable of transmitting receptor signals similarly to $G_s\alpha$ although its biological roles are still being defined. The XLαs DMR also generates antisense (NESPAS) transcripts that are important for establishing NESP55 imprinting.

AHO is caused by heterozygous mutations involving $G_s\alpha$ exons 1–13 or their splice junctions that either disrupt mRNA or protein expression or alter the amino acid sequence to produce inactive protein.[1] PHP1A and PPHP patients within the same kindred have identical mutations on the maternal and paternal alleles, respectively. Mutations occurring in exon 1 are consistent with deficiency of $G_s\alpha$, rather than other alternative *GNAS* products, underlying the AHO and PHP1A phenotypes.[29] A mutation within the alternatively spliced exon 3 was reportedly resulting in loss of expression of the long form of $G_s\alpha$ and leading to normocalcemic PHP1A with no obesity.[30] A specific 4 bp deletion in exon 7 has been identified in many families, probably resulting from DNA polymerase pausing and slipped strand mispairing.[31]

Generally, most mutations are complete null mutations, and there is little obvious genotype–phenotype correlation, although one study reported that subcutaneous ossifications were more extensive in patients with truncating as compared to missense mutations.[32] Several missense mutations have been shown to have specific effects on $G_s\alpha$ function.[1] Mutation of either the basic residue Arg231 within switch 2, or the acidic residue Glu259 within switch 3 results in a receptor-activation defect, probably by disrupting interactions between these two switch regions that stabilize the active conformation. The Ala366Ser mutation results in PHP1A plus gonadotropin-independent precocious puberty (testotoxicosis) in males. At core body temperature, the mutant protein is thermolabile resulting in PHP1A; whereas at the lower testicular temperature the mutant protein is stable, but is constitutively activated due to increased basal GDP release. Mutations at the carboxyl terminus prevent $G_s\alpha$ from being activated by receptors.

PHP1B is caused by absence of maternal-specific methylation of the exon 1A DMR resulting in a paternal-specific imprinting pattern (unmethylated, transcriptionally active) on both alleles.[33] In sporadic cases, the NESP55 and XLαs/NESPAS DMRs are also typically

associated with abnormal methylation resulting in the maternal allele having a paternal methylation pattern throughout the *GNAS* locus. This broad *GNAS* imprinting defect in sporadic cases can also be sometimes associated with imprinting defects in other imprinted genes (multilocus imprinting disturbance).[34–38] It is unlikely that the *GNAS* imprinting defects in sporadic PHP1B are due to an underlying mutation in the vicinity of *GNAS*.[39,40] PHP1B with broad *GNAS* methylation defects more rarely results from paternal uniparental disomy (pUPD).[41,42]

Most commonly AD-PHP1B (familial) is associated with a deletion within the linked *STX16* (syntaxin 16) gene and a *GNAS* methylation defect involving only the exon 1A DMR.[33,43,44] Maternal transmission of this deletion leads to PHP1B; whereas paternal transmission results in a silent carrier. Less commonly, AD-PHP1B is associated with maternal deletions involving NESP55[45–47] or NESPAS[48] resulting in a switch of the maternal allele to the paternal methylation pattern throughout *GNAS* locus. PHP1B has also been reported to be associated with duplications within the GNAS upstream region in two unrelated patients.[49] There is no significant correlation between the extent of *GNAS* methylation defects at upstream NESP55 and XLαs DMRs and clinical presentation.[50,51] Three siblings with PHP1B had a 3 bp deletion in exon 13 (ΔI382) that deletes $G_s\alpha$ Ile382.[52]

2.4 Animal Models

Many of the mouse models involving the mouse ortholog *Gnas* have been previously reviewed in detail.[53] Models disrupting $G_s\alpha$ expression have confirmed that $G_s\alpha$ is imprinted in a tissue-specific manner and the renal PTH resistance develops after maternal, but not paternal, transmission of the mutation. It was shown that $G_s\alpha$ imprinting in mouse proximal tubules is not present at birth, but established during early postnatal development, which may explain the delayed onset of renal PTH resistance in PHP1A.[54] While $G_s\alpha$ is preferentially expressed from the maternal allele in renal proximal tubules, it is biallelically expressed in most other tissues, including skeletal muscle, liver, spleen, lung, and renal medulla. Lack of imprinting in the renal medulla may explain why the mice have no long-term problems with vasopressin sensitivity and water balance. Another feature in common with AHO found in two of the mouse models is heterotopic ossification.[55,56] Mice with maternal $G_s\alpha$ disruption also develop other features not found in AHO, including subcutaneous edema at birth and significant early lethality. Loss of XLαs in mice leads to early postnatal loss and a severely lean, hypermetabolic phenotype.[57,58] However, it is unclear how relevant this finding is to humans, as PPHP patients with paternal *GNAS* mutations that should disrupt XLαs do not develop this phenotype.

Maternal, but not paternal, $G_s\alpha$ mutations in mice lead to severe obesity (which mimics the inheritance pattern of obesity in AHO patients), diabetes, insulin resistance, and hyperlipidemia.[53] The obesity in maternal $G_s\alpha$ knockout mice is primarily due to reduced sympathetic nervous system (SNS) activity and energy expenditure rather than increased food intake. This parent-of-origin effect on metabolism was reproduced in mice in which $G_s\alpha$ disruption was limited to the central nervous system.[28] In this model, maternal, but not paternal, $G_s\alpha$ deletion in the central nervous system impaired the ability of a central melanocortin agonist to stimulate energy expenditure, but did not affect the ability of the agonist to acutely reduce food intake. Central melanocortin effects on food intake appear to be primarily mediated via $G_{q/11}\alpha$ rather than $G_s\alpha$.[59] The obesity and reduced energy expenditure associated with maternal $G_s\alpha$ mutations results from $G_s\alpha$ imprinting at a central nervous system site outside of the paraventricular nucleus of the hypothalamus.[60]

Deletion of the exon 1A DMR on the paternal allele reversed $G_s\alpha$ tissue-specific imprinting (silencing of the paternal allele) and resulted in increased renal PTH sensitivity due to increased $G_s\alpha$ expression in renal proximal tubules.[53] In addition, this deletion reverses all the metabolic consequences resulting from maternal $G_s\alpha$ deletion.[61] This finding confirms the critical role this region plays in $G_s\alpha$ imprinting and helps to explain the importance of this region to the pathogenesis of PHP1B. An *STX16* deletion in mice analogous to the one associated with AD-PHP1B did not replicate the exon 1A DMR methylation defect or PTH resistance,[62] while the AD-PHP1B-associated NESP55 deletion in mice was able to reproduce the methylation defects and PTH resistance observed in patients, although these mice had severe early postnatal lethality and hypoglycemia associated with excess expression of XLαs.[63,64]

2.5 Functional and Molecular Pathology

Tissue-specific $G_s\alpha$ imprinting underlies the differential clinical effects (multihormone resistance and obesity) of maternal versus paternal $G_s\alpha$ mutations in PHP1A and PPHP patients, respectively.[1] In specific hormone target tissues, where $G_s\alpha$ is primarily expressed from the maternal allele (e.g., renal proximal tubules for PTH, thyroid, pituitary, and gonad), a null mutation on the active maternal allele disrupts $G_s\alpha$ expression and hormone signaling, whereas the same mutation on the inactive paternal allele has little effect on $G_s\alpha$ expression or hormone signaling (Fig. 35.4A). Consistent with this, PTH-stimulated urinary cAMP is markedly reduced in PHP1A, but remains normal in PPHP. In most other nonendocrine tissues, $G_s\alpha$ is not imprinted and therefore both maternal and paternal $G_s\alpha$ mutations lead to

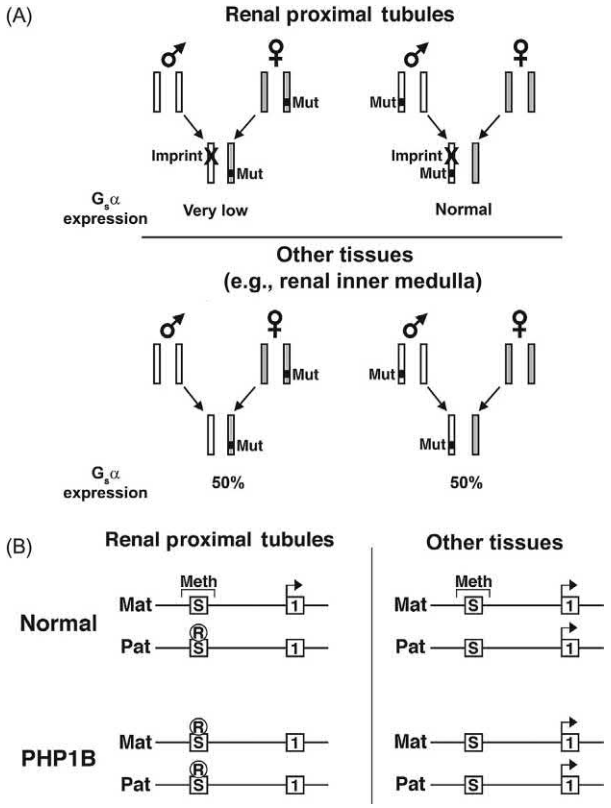

FIGURE 35.4 Tissue-specific $G_s\alpha$ imprinting and its role in PHP1A and PHP1B. (A) Influence of tissue-specific $G_s\alpha$ imprinting in the pathogenesis of PHP1A/AHO. In renal proximal tubules (above), $G_s\alpha$ is paternally imprinted (denoted with an X). Mutation (Mut) of the active maternally inherited allele *(gray rectangle)* in PHP1A leads to loss of $G_s\alpha$ expression (left), whereas mutation of the inactive paternal allele in PPHP has little effect on $G_s\alpha$ expression (right). In most other tissues (below) $G_s\alpha$ is not imprinted and therefore mutation of either the maternal and paternal alleles leads to similar 50% loss of $G_s\alpha$ expression (haploinsufficiency). (B) Model for $G_s\alpha$ tissue-specific imprinting and the pathogenesis of PHP1B. In the model shown binding of a tissue-specific *trans*-acting repressor (R) to a silencer (S) within the exon 1A DMR silences the $G_s\alpha$ promoter. Normally (above) in renal proximal tubules the repressor binds to the silencer on and inhibits $G_s\alpha$ expression from the paternal allele, but is unable to bind to the maternal allele due to methylation, allowing the maternal $G_s\alpha$ promoter to remain active. In most other tissues the repressor is not expressed, and therefore $G_s\alpha$ is expressed from both parental alleles. In PHP1B (below) maternal-specific methylation of the exon 1A DMR is absent, allowing the repressor to bind to and suppress the $G_s\alpha$ promoter on both parental alleles leading to $G_s\alpha$ deficiency and PTH resistance in renal proximal tubules. In most other tissues loss of exon 1A DMR methylation does not affect $G_s\alpha$ expression, as the repressor is not expressed.

a similar 50% reduction in expression in many tissues in PHP1A and PPHP patients, respectively.

Loss of PTH signaling in renal proximal tubules due to $G_s\alpha$ deficiency in PHP1A (and PHP1B) impairs both the conversion of 25-hydroxyvitamin D to $1,25(OH)_2D$, and the reabsorption of phosphate. Low $1,25(OH)_2D$ levels lead to reduced intestinal calcium absorption and skeletal calcium mobilization. Decreased phosphate excretion leads to hyperphosphatemia, which further inhibits $1,25(OH)_2D$ production. All of these effects combined result in hypocalcemia, hyperphosphatemia, and secondary hyperparathyroidism.

Hypothyroidism, hypogonadism, and growth hormone deficiency in PHP1A result from TSH, gonadotropin, and GHRH resistance due to partial $G_s\alpha$ deficiency in thyroid, gonads, and pituitary somatotrophs, respectively.[1] Gonadotropin levels are not clearly elevated in PHP1A and therefore it was proposed that these patients have a partial resistance that allows for follicular development and estrogen production, but not ovulation.[4] PHP1A patients lack clinical resistance to other hormones that activate $G_s\alpha$ (e.g., ACTH and vasopressin). One possible explanation for this is that $G_s\alpha$ is not imprinted in their respective target tissues (adrenals and distal nephron) and therefore mutations lead to only a 50% reduction in $G_s\alpha$, which may still allow enough cAMP generation to elicit the physiological response to these hormones. Lack of $G_s\alpha$ imprinting may also explain why the anticalciuric action of PTH in the thick ascending limb is unaffected in PHP1A.[65]

The obesity associated with PHP1A, but not PPHP, presumably results from $G_s\alpha$ deficiency in one or more metabolically active tissues, where $G_s\alpha$ is imprinted due to the combined effects of mutation of the active maternal allele and silencing of the paternal allele. Liver, muscle, and adipose tissue are unlikely to mediate these effects, as there is no evidence for $G_s\alpha$ imprinting in these tissues.[53] Studies in mice show that the parent-of-origin effect of $G_s\alpha$ mutations on energy balance is mediated in the central nervous system due to imprinting in one or more brain regions.[28] This effect appears to result from selective resistance to central melanocortins, which activate $G_s\alpha$, resulting in reduced SNS activity and energy expenditure, but no primary effect on food intake. This model is consistent with the finding of reduced serum norepinephrine levels and resting energy expenditure in PHP1A patients,[66–68] and the report of a PHP1A infant who developed early-onset obesity without overeating.[69] $G_s\alpha$ imprinting in brain may also account for the greater burden of neurocognitive problems in PHP1A, as compared to PPHP.[23]

Features of AHO common to both PHP1A and PPHP are almost certainly the consequence of $G_s\alpha$ haploinsufficiency resulting from heterozygous mutation in tissues, where $G_s\alpha$ expression is not affected by imprinting. Studies in mice suggest that brachydactyly in AHO results from impaired local action of PTHrP on growth plate chondrocytes leading to their accelerated differentiation and to long bone shortening.[1,70,71] Ectopic ossifications in AHO and POH occur by intramembranous ossification, as reduced cAMP promotes osteoblast differentiation and expression of the osteoblast-specific factor Cbfa1/RUNX2.[1] It is unclear why many POH patients do not have other AHO features or why there is a

predilection for paternal transmission of $G_s\alpha$ mutations in POH patients.

PHP1B is not associated with typical $G_s\alpha$ inactivating mutations found in PHP1A; and in contrast to PHP1A, PHP1B is generally not associated with a large decrease in $G_s\alpha$ expression or bioactivity in accessible tissues (e.g., blood cells) or the AHO phenotype.[1] Rather PHP1B results from loss of maternal-specific methylation of the exon 1A DMR leading to a paternal-specific imprinting pattern (unmethylated) on both parental alleles.[1] AD-PHP1B deletion mutations implicate regions in *cis* within (NESP55 and NESPAS) or outside of the *GNAS* locus (*STX16*) that are important for the establishment or maintenance of maternal-specific *GNAS* methyation patterns. Broad *GNAS* methylation changes on the maternal allele may also be associated with sporadic PHP1B or result from pUPD.

Loss of exon 1A DMR methylation in PHP1B implicates this region in tissue-specific $G_s\alpha$ imprinting. This likely involves both exon 1A DMR maternal-specific methylation, which is normally present in all tissues, and a tissue-specific *trans*-acting factor that can bind to the unmethylated paternal exon 1A DMR and suppress the paternal $G_s\alpha$ promoter.[1] For example, the exon 1A DMR may contain a silencer or boundary element that binds a tissue-specific repressor or insulator protein on the paternal allele, but which is unable to bind the tissue-specific factor on the maternal allele due to its being methylated (Fig. 35.4B). The paternal $G_s\alpha$ promoter is suppressed only in tissues where the tissue-specific factor is expressed (e.g., renal proximal tubules), whereas most other tissues it is biallelically expressed. In PHP1B both alleles are unmethylated, allowing the tissue-specific factor to bind to and suppress $G_s\alpha$ expression from both alleles in renal proximal tubules. $G_s\alpha$ expression would remain biallelic in other tissues, where the factor is not expressed. This model is consistent with the presence of $G_s\alpha$ deficiency in renal proximal tubules, but not in other tissues, in PHP1B patients, and is supported by the observation that paternal exon 1A DMR deletion reverses $G_s\alpha$ imprinting in mice.[1] Occasionally, patients with the methylation defect of PHP1B may have features of AHO, particularly brachydactyly,[72,73] and this may correlate with the extent of tissue-specific $G_s\alpha$ imprinting.[74]

2.6 Diagnosis

The diagnosis of PHP is suggested by the presence of hypo- or eucalcemia, hyperphosphatemia, and elevated PTH in the absence of renal insufficiency or vitamin D deficiency. The gold standard to confirm PHP due to a $G_s\alpha$ defect (PHP1A or PHP1B) is to demonstrate an impaired urinary cAMP response to PTH analog (Ellsworth–Howard test). However, this test is generally not clinically necessary and the analog is not presently commercially available.

Many features of AHO are nonspecific and therefore the diagnosis cannot be made based solely on the presence of physical signs and symptoms. Features of AHO (e.g., brachydactyly, obesity, and neurocognitive deficits) can also be seen in other genetic disorders (e.g., Prader–Willi, brachydactyly syndromes, Turner's syndrome, Rubinstein–Taybi syndrome, 2q37 deletion, acrodysostosis). Osteoma cutis and PTH resistance are much more specific for AHO and therefore should increase the suspicion of this diagnosis. AHO features in the absence of family history or hormone resistance requires confirmation of a $G_s\alpha$ defect by biochemical or genetic studies to establish the diagnosis. The initial test would be *GNAS* mutation screening by commercial laboratory, which is ~70% sensitive. The diagnosis can also be confirmed by documenting an ~50% loss of erythrocyte $G_s\alpha$ bioactivity or expression levels, which are performed in research laboratories. Bioactivity assays are typically performed by stimulating G_s with a nonhydrolyzable GTP analog. However, patients who have mutations that specifically disrupt receptor coupling have normal results in this assay and are misdiagnosedas having PHP1C.[75] Therefore, these assays should be performed using a receptor ligand, such as isoproterenol. *GNAS* methylation analysis can be performed in patients with PTH resistance, AHO features, and no apparent $G_s\alpha$ defect, but a maternal deletion, including the exon 1A DMR, must be ruled out in those who are positive in the methylation analysis. Acrodystosis is a congenital disorder that mimics AHO, but with much more severe and widespread skeletal defects. Recently, mutations in the protein kinase A regulatory-1α subunit have been identified in patients with acrodysostosis and multihomone resistance, a condition that mimics PHP1C.[76] Patients with ectopic ossifications characteristic of POH should be examined for features of AHO and biochemically screened for multihormone resistance.

PHP1B is diagnosed by the presence of renal PTH resistance in the absence of AHO or other hormone resistance (except for mild TSH resistance). These patients generally have no clear reduction in erythrocyte $G_s\alpha$ expression or bioactivity. The diagnosis is confirmed by exon 1A DMR methylation analysis of blood genomic DNA. Genetic testing for *STX16*, NESP55, or NESPAS deletions can also be performed in research laboratories to confirm the diagnosis of AD-PHP1B. In rare patients with normal exon 1A DMR methylation, genetic screening for the ΔIle382 mutation can be performed.

2.7 Counseling

AHO patients (both PHP1A and PPHP) should be counseled that there is a 50% chance of each offspring developing AHO. Females need to be counseled that

their affected offspring will also have multihormone resistance and obesity and potentially significant neurocognitve problems, while males should be counseled that hormone resistance and early-onset obesity is unlikely to occur in their offspring. As the AHO phenotype is variable, it is impossible to predict its severity in offspring and patients need to be told that their affected offspring may have severe physical and neurocognitive manifestations, including POH, even if their features are mild. Male POH patients should be counseled that each of their offspring has a 50% chance of having PPHP or possibly POH. Female POH patients should be counseled that each of their offspring has a 50% chance of developing PHP1A and a chance of developing POH. If a *GNAS* mutation has been identified, then genetic testing will be useful in identifying further affected family members and could be theoretically used for prenatal testing.[77]

In AD-PHP1B, PTH resistance only occurs in offspring who inherit the trait maternally with a 50% rate of transmission from affected females. Offspring of affected males who inherit a PHP1B-associated mutation will be clinically silent carriers, who may themselves pass on PHP1B if they are female. Serum calcium, phosphorus, and PTH should be serially measured beginning in childhood in offspring who inherit the *STX16* deletion from their mother. Alternatively, family members could be screened for the *GNAS* 1A methylation defect to determine who is at risk for developing PTH resistance. While prenatal diagnosis could be made by analysis of fetal genomic DNA, this is probably not indicated given the limited and easily treatable manifestations of the disorder.

2.8 Treatment

There is no specific therapy for the physical and neurocognitive manifestations of AHO. The ossifications in either AHO or POH do not require surgical excision unless they are causing discomfort or disfigurement. PTH resistance in PHP1A and PHP1B should be treated aggressively with oral calcium and vitamin D (either high dose ergo- or cholecalciferol or calcitriol at more physiologic doses). Unlike patients with primary hypoparathyroidism, PHP patients generally do not develop hypercalciuria with treatment, as the calcium-reabsorbing effect of PTH in the renal distal tubule is maintained.[65] Therefore, the goal of therapy is to normalize both calcium and PTH levels, if possible, while monitoring for hypercalciuria. Normalizing PTH is important for preventing the skeletal consequences of high circulating PTH levels (particularly important in PHP1B) and the development of autonomous parathyroid tumors leading to tertiary hyperparathyroidism.[78] Rarely, patients will require surgical excision of these tumors. Cinacalcet has also been successfully used in two PHP1B patients with severe hyperparathyroidism.[78,79] TSH and gonadotropin resistance in PHP1A is treated with levothyroxine and oral contraceptives (in females) or testosterone (in males), respectively. GH therapy may be beneficial in PHP1A patients with GH deficiency.[80]

3 FIBROUS DYSPLASIA/MCCUNE–ALBRIGHT SYNDROME

3.1 Brief Clinical Description

The original descriptions of what has come to be called the McCune–Albright syndrome (MAS) by Donovan McCune and Fuller Albright established the triad of osteitis fibrosa (fibrous dysplasia of bone, FD), café-au-lait macules of the skin, and sexual precocity as defining the disease.[81,82] However, the case described by McCune, which included the presence of hyperthyroidism, and subsequent cases revealed that manifestations of MAS could include other endocrinopathies, such as growth hormone excess (gigantism/acromegaly),[83] cortisol excess (Cushing's syndrome),[84] and hypophosphatemia.[85] In addition, a number of less common clinical findings have also been seen in the context of MAS and represent less common manifestations of the disease. These include intramuscular myxomas (Mazabraud's syndrome),[86] a form of neonatal hepatitis,[87] cardiac involvement,[88] gastrointestinal involvement that includes pancreatic intraductal papillary muscinous neoplasms[89] and polyps,[90] platelet dysfunction,[91] and others. Indeed, the spectrum of the clinical manifestations of McCune–Albright syndrome is as broad as the tissue distribution of $G_s\alpha$ expression. Several malignancies, including thyroid,[92] breast,[93,94] bone,[95] and testicular[96] have been found in association with MAS. The relative prevalence of the manifestations of MAS seen in the large cohort of patients studied at the National Institutes of Health can be found in Table 35.1.

FD involving a single bone (monostotic FD, MFD) has been shown to harbor $G_s\alpha$ mutations[97] and is probably the most common form of a $G_s\alpha$ disease. As such, it can be considered a forme fruste of MAS. Polyostotic FD (PFD) is less common than MFD, and MAS is the least common. The relative prevalence of each is not known. Generally, the younger the age of presentation the more extensive the skeletal and/or extraskeletal involvement. The sites most commonly affected are the skull base and proximal femora.[98] Monomelic disease is more common; and with PFD, there is a generally a proximal to peripheral gradient, with distal limb disease almost always accompanied by more extensive proximal disease. Clinically insignificant disease is sometimes discovered incidentally in adulthood during the evaluation of some other medical condition.

TABLE 35.1 Prevalence of Major Findings in the NIH Cohort of Patients With Fibrous Dysplasia/McCune–Albright Syndrome

Clinical finding	% Patients[a]
Fibrous dysplasia	98
Café-au-lait spots	66
Gonadal abnormalities	
Male: (ultrasound)[b]	70
Female: sexual precocity	50
Thyroid abnormalities	
Abnormal ultrasound (U/S)	66
Hyperthyroid + abnormal U/S	28
Renal phosphate wasting	43
Hypophosphatemia	10
Growth hormone excess	21
Cushing's syndrome	4

[a] n = *140; 58 Males, 82 females.*
[b] *Detected on ultrasound,* 21% *of all males had precocious puberty.*

FD involving the long bones typically presents in childhood with limp, pain, fracture, or deformity. The clinical course is dependent upon the extent of the skeletal disease burden, with more extensive disease more likely to lead to repeated fractures, chronic pain, deformity, and in severe cases impaired function, including the need for ambulatory aids.[99] The more extensive the skeletal burden of FD, the more likely it is that the FD will be accompanied by renal phosphate wasting, osteomalacia, and rickets, which is due to overproduction of the phosphaturic hormone, fibroblast growth factor 23 by the FD tissue.[100,101]

Involvement of the axial skeleton, including the craniofacial structures is common. Craniofacial FD (CFD) often presents with an asymptomatic, asymmetric deformation. The major issue related to CFD is cosmesis. Functional impairment due to CFD, primarily vision and/or hearing loss due to compression of neural structures, is uncommon. Functional impairment, as well as significant deformity, is much more commonly seen in association with GH excess. While there are dental findings seen in association with FD, in general the dental arch is preserved and common dental procedures can be performed without FD impacting on the outcome. Spine disease is common, and can often be associated with scoliosis.[102] Untreated scoliosis is one of the few aspects of FD/MAS that is associated with early mortality.

3.2 Genetics Description

With the exception of a single report of a mother and daughter with features compatible with MAS, inheritance has not been reported.[103] Instead, MAS and FD appear to be caused by somatic mutation of *GNAS* with germline transmission presumed lethal.

3.3 Molecular Genetics

In vitro studies have shown that $G_s\alpha$ residues R201 and Q227 are critical for its intrinsic GTPase activity and that mutation of either of these residues leads to constitutive activation by decreasing the normal GTPase activity (Fig. 35.1), with perhaps Q227 mutations having a more pronounced effect.[1] Greater than 95% of all published reports of FD/MAS in which mutation testing has been performed have identified mutations at the R201 residue,[104] the vast majority being either histidine (His) or cysteine (Cys). However, infrequently, arginine is replaced by serine, glycine, or leucine. Rarely, mutations of Q227 residue to Arg can be found.[105] A mosaic distribution of the $G_s\alpha$ mutation is supported by the lack of vertical transmission, and the presence of *café-au-lait* skin macules along the developmental lines of Blaschko in MAS. The extent and distribution of mutant-bearing cells is a function of the timing of the mutation during development and will dictate the extent of tissues clinically affected in the patient. The most severely affected MAS patients have mutations in tissues derived from all three germ layers (ectoderm, mesoderm, endoderm), suggesting that the somatic mutation occurs very early in postimplantation development. Mutations affecting only a single organ or tissue presumably occur later in development. Focal occurrence of $G_s\alpha$ mutation has been described in MFD, in isolated endocrine tumors of the pituitary and thyroid, and even in Sertoli cells.[106]

The complex transcriptional regulation of the *GNAS* gene, including tissue-specific differences in imprinting of several gene transcripts, also influences clinical expression of activating $G_s\alpha$ mutations. Pituitary tumors, particularly somatotrophs, appear to harbor $G_s\alpha$ mutations exclusively on the maternal allele.[107] The XLαs transcript has also been shown to activate cAMP formation constitutively when bearing the equivalent R201H mutation and may, in certain cases of MAS and FD, be responsible for molecular pathogenesis when the mutation occurs on the paternal allele.[108]

3.4 Animal Models

Mouse models leading to constitutively activation of cAMP formation have been created by transgenic overexpression of $G_s\alpha$, by expression of R201H or Q227L mutant forms of $G_s\alpha$, or by expression of the cholera toxin A1 subunit, which covalently modifies R201 (Fig. 35.1). Transgenic expression of the cholera toxin A1 subunit in somatotrophs leads to pituitary hyperplasia and gigantism, whereas expression in thyroid cells leads to thyroid hyperplasia and hyperthyroidism.[109] $G_s\alpha$ overexpression

in the heart leads to cardiomyopathy,[110] and expression of constitutively-activated forms of $G_s\alpha$ in the forebrain disrupts associative and spatial learning.[111] A model of FD was created by transplanting $G_s\alpha$-mutated skeletal progenitor cells into immunocompromised mice.[112] A mouse model with germline expression of the R201C mutation survived, and with aging developed a skeletal dysplasia radiographically and histologically similar to FD.[113]

3.5 Functional and Molecular Pathology

The pathological hallmark of FD is the expansion of a population of fibroblast-like cells that have molecular and cell surface markers of cells of the osteogenic lineage.[114,115] These cells have been shown to be derived from skeletal stem cells,[116] sometimes referred to as mesenchymal stem cells. In FD $G_s\alpha$ activation and resultant excess cAMP, via as yet undefined downstream pathways, lead to proliferation of a population of cells arrested in an early stage of osteogenic cell differentiation and inhibition of differentiation to mature, bone-forming osteoblasts. At the tissue level, FD is also a mosaic disease, as lesions are composed of both normal and mutation-bearing cells.[112] The percentage of cells carrying the $G_s\alpha$ mutation varies from site to site, and with age. In FD tissue from older patients, there are few cells that harbor the $G_s\alpha$ mutation.[117] This loss of mutation-bearing cells occurs in the context of massive apoptosis seen in the FD of younger patients, suggesting that the $G_s\alpha$ mutation in skeletal stem cells leads to proliferation, inhibition of differentiation, and an age-dependent apoptosis.

Both hyperplasia and adenoma have been described in affected endocrine tissues in MAS, as well as isolated endocrine tumors harboring $G_s\alpha$ mutations. There are no specific pathognomonic features distinguishing such endocrine tumors from benign endocrine tumors of other molecular pathogenesis.

3.6 Diagnostic

While molecular testing for $G_s\alpha$ mutations that cause MAS/FD are available, the mainstay of diagnosis is clinical. This owes in part to the fact that FD/MAS is a mosaic disease, and reliability of testing is greatest when it is performed on affected tissue. Furthermore, within affected tissue the percentage of cells harboring the mutation may be small and standard mutation testing techniques may not be sensitive enough to detect a low level of mutant alleles. In these cases, techniques that selectively amplify the mutant allele and suppress amplification of the wild type allele may be helpful.[118] Mutation testing is most often positive when the disease is obvious on clinical grounds, and thus not necessary.[119] As such, the results of mutation testing rarely change clinical management. Mutation testing is most helpful in excluding FD as the diagnosis in the broad array of non-FD fibroosseous lesions of the craniofacial bones that have radiographic and histopathological features reminiscent of FD, but behave differently clinically. In these cases, when the only potential disease manifestation is a skeletal lesion in the craniofacial bones, negative mutation testing is supportive in excluding FD as the diagnosis.

The diagnosis of FD is made principally on the basis of the radiographic appearance, the anatomical location and distribution, and is made easier when other features of MAS are present. The radiographic appearance of FD has been classically described as having a ground glass appearance (Fig. 35.5A and C). While this is usually the case, especially in children, which is when the majority of patients are diagnosed, in older patients FD lesions may be dominated by a more sclerotic and less homogenous appearance (Fig. 35.5B–C). The same phenomenon of an age-related change in the radiographic appearance of FD is also true with CFD, again with young patients demonstrating a homogeneous ground glass appearance and older patients a mixed lytic and sclerotic appearance (Fig. 35.5D–F). Skull base and proximal femur involvement, as well as asymmetric involvement, are suggestive of the diagnosis of FD.

If uncertainty exists in the diagnosis, biopsy and histology can be helpful. Microscopically FD lesions have what has been classically defined as a Chinese character appearance (Fig. 35.5G). An additional histopathological feature, which can be detected on mineral stains of undecalcified specimens, and which contributes to the plasticity of the material property of FD and its propensity to deform, is the osteomalacic nature of FD (Fig. 35.5H). In addition to the fact that there are differences in the histopathological appearance of FD depending on the anatomical site from where it was derived (CFD tends to have a more sclerotic/hypercellular appearance[120]), even within the same patient one can find a spectrum of histopathological findings (Fig. 35.5I). If uncertainty persists, mutation testing on affected tissue can be performed.

The diagnosis of FD at a single anatomical site necessitates the need to assess for FD at other sites. While a skeletal survey may identify most FD lesions, disease at some sites, such as the skull, spine, and ribs, can be missed. For this reason, a nuclear medicine bone scan at the time of diagnosis is recommended as the best imaging modality for determining the extent of skeletal involvement. The "map" of anatomically involved sites is established early in life, with the vast majority of clinically significant disease established by the age of 5.[121] However, before the age of 5 even a nuclear medicine bone scan may be insensitive at detecting sites of what will eventually become active FD.

All patients with FD should be screened for endocrine abnormalities.[122] The most frequent endocrine dysfunction

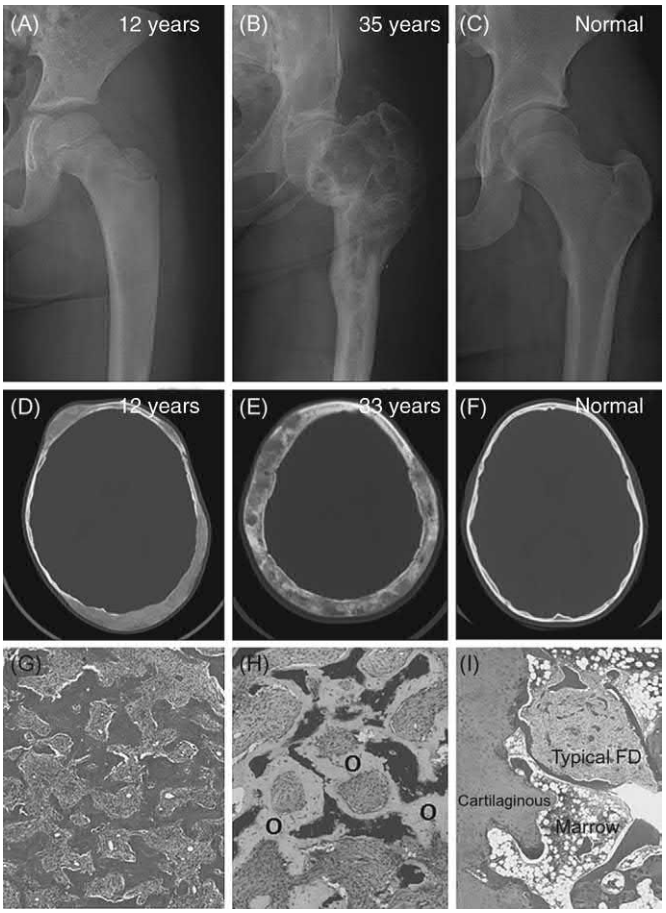

FIGURE 35.5 Radiographic and histologic appearance of fibrous dysplasian (FD). (A) Radiograph of FD in the left femur of a young patient (12 years old) showing what is typically described as "ground glass" appearance. (B) Less homogeneous appearance with mixed areas of sclerosis and lysis seen in the radiograph of an FD lesion from a 35-year-old. (C) Radiograph of a normal femur shown for comparison. (D) Similar "ground glass" appearance observed in the craniofacial bones of a 12-year-old patient as is seen in this axial image of a CT of the skull. (E) Similar conversion from the homogeneous "ground glass" appearance observed in younger patients to the mixed sclerotic and lytic lesions in older patients is seen in the CT of this 33-year-old patient. (F) A scan from a normal control shown for comparison. The relatively thinness of normal skull bone highlights the tremendous thickening of the skull that can occur in FD. (G) The classic "Chinese writing" appearance of FD is demonstrated in this H&E-stained section of demineralized bone, which features replacement of hematopoetic marrow with a fibrous tissue composed of relatively undifferentiated cells of the osteogenic lineage. (H) Von Kassa-stained FD section showing osteomalacic bone with unmineralized osteoid (O) showing as light gray and mineralized bone staining black. (I) Section showing that FD can sometimes be a heterogeneous tissue that includes areas of typical FD, areas of normal appearing bone marrow, and areas of cartilage-like material.

seen in girls with FD/MAS is sexual precocity. Vaginal bleeding, usually preceded by the development of breast tissue, without signs of virilization is the most common presentation. An ultrasound of the ovaries will show cysts. At presentation, cystic lesions are usually found on one side, but not infrequently bilateral involvement can be seen. Gonadotropins are suppressed and do not rise in response to stimulation with gonadotropin-releasing hormone (GnRH) analogues, confirming the gonadotropin-independent nature of the precocious puberty seen in MAS.[123] Sexual precocity is less common in boys, even though testicular involvement as detected by ultrasound is common (seen in > 50% of the males).[96] Boys present with signs of virilization and have gonadotropin levels that are low and not stimulated with GnRH analogues.

Thyroid disease is common, and while it can present with obvious signs of hyperthyroidism, such as a goiter, it is often "subclinical." There are typical ultrasonographic findings, TSH is suppressed, and $G_s\alpha$ activation leads to a T3-dominant picture on thyroid function tests.[124] Cushing's syndrome only occurs in the neonatal period with a Cushingoid appearance, café-au-lait macules, and elevated and nonsuppressible serum cortisol. These patients often have multisystem involvement (thyroid, liver, and other), and must be diagnosed and treated early and aggressively to avoid early death.[125] Renal phosphate wasting is common and its severity correlates with the skeletal burden of FD. It is easily detected by a simple measurement of serum phosphorus.[100] It is important to diagnose and treat both hypophosphatemia, as well as other endocrinopathies, as they can worsen the FD.[126] The diagnosis of GH excess can sometimes be obvious, manifesting with tall stature, although it is sometimes confounded by the presence of sexual precocity, in which case it is masked or mistakenly ascribed to the tall stature seen in the early stages of precocious puberty. GH excess can also be subtle, with growth velocity only slightly increased. A high index of suspicion and measurement of serum GH, IGF-1, and performing an oral glucose tolerance test is necessary.[127,128]

3.7 Counseling

Consideration of how to counsel patients with FD/MAS is framed by cognizance of the fact that FD/MAS is a sporadic, mosaic disease, and that the mutation does not survive in the germ cells. Given the rarity of the disease antenatal testing is not practical. Mosaicism and the assumption that germ line mutations are lethal allow one to be able to assure patients that they will not transmit the disease to their offspring. Perhaps, one of the most important aspects of counseling parents of children with FD/MAS has to do with our current understanding of the fact that the "map" of affected tissues is established very early in development, and that this results in the establishment of affected (and equally importantly unaffected) tissues very early in life. As such, at the time of presentation, all affected and unaffected tissues can be identified with appropriately thorough testing in most cases. This allows for the identification of problem areas, as well as the identification of what

tissues are not affected, and will likely never be affected. In many cases, the latter point is a source of tremendous relief for many parents. The ability to identify affected and unaffected tissues at an early age, of course, necessitates a thorough baseline evaluation. Once any aspect of FD/MAS is diagnosed, it is prudent to perform testing of all tissues potentially affected to identify and exclude affected tissues. In most cases, with appropriate testing, all affected and unaffected tissues can be identified by the age of five.

3.8 Treatment

Surgery remains the mainstay of treatment for FD of the appendicular skeleton. Indications for surgery include the correction of deformity that is interfering with ambulation, management of recurrent fractures, nonunion of fractures, transformation of the lesions into either aneurysmal bone cysts or malignant tumors, and chronic weight-bearing bone pain not relieved by medical management.[129] Surgery, however, is never curative, and reoperation is frequently necessary. The length of time between operations is dependent upon the anatomical site in question, the extent of the disease at the involved site, the degree of tissue osteomalacia, and the age and activity level of the patient. Extensive disease of the proximal femur (one of the most commonly affected anatomical sites) in a young patient with hypophosphatemia may require surgery every 18–24 months during childhood. The approach to surgical care has evolved over the years. While early reports advocated techniques that generally work well for isolated skeletal lesions, such as curettage, grafting, and the use of external fixation,[130] more recently it has been recognized that these approaches are not optimal for the care of FD. With curettage and grafting, the graft is almost universally resorbed, especially in the active lesions of young persons, and intramedullary rods are generally felt to be superior to external fixation devices.[129,131,132]

As opposed to the appendicular skeleton, surgery is rarely indicated in treatment of CFD. Indications for treatment of CFD include documented deterioration of vision or hearing, and/or cosmesis. Management of skull base FD has been controversial. The optic nerves course through the skull base, which is the most commonly involved anatomical site. It had been previously suggested that when the optic nerves were encased with FD vision loss was inevitable. However, recent studies that have included relatively large numbers of patients followed over a relatively long period of time found vision loss to be uncommon with expectant management.[133,134] Vision loss was much more common in subjects following prophylactic operations to prevent vision loss, or in the subgroup of patients with growth hormone excess.[135] The latter point highlights the role of endocrine dysfunction exacerbating FD. Growth hormone excess is clearly associated with vision loss, as well as overgrowth and the potential for massive dysmorphism in CFD.[127,128] Additionally, inadequately treated hyperthyroidism and/or hypophosphatemia are associated with earlier and more frequent fractures,[101] as well as histological changes known to be associated with poor bone quality.[126]

Pain is one of the most common and difficult clinical problems associated with FD. While pain is one of the indications for surgery, it should not be undertaken until medical management has failed after an optimal trial. A stepwise approach should be taken in treating pain associated with FD. The first step includes typical, nonnarcotic analgesics. This is followed by bisphosphonates, which have been shown to be very effective in controlling FD-associated pain.[136] If bisphosphonate treatment fails, surgery should be considered, but if surgery is not felt to be feasible or clinically helpful, narcotic analgesics should be considered. This is not infrequently the only successful option for some patients.[137]

While there was great hope that bisphosphonates would be an effective medical treatment that would prevent expansion and improve bone quality, this has not proven to be the case. The best prospective study of this failed to show an effect on expansion or an effect at the tissue level that suggested of improved bone quality,[138] and a recently published placebo controlled trial failed to show clear benefit of oral alendronate.[139] Two new drugs that hold hope for an effective treatment for FD based on their mechanism of action and an understanding of the biology of FD are the anti-IL-6 receptor antibody, tocilizumab, and the anti-RANKL antibody, denosumab.[140] Trials are underway that will eventually answer the question as to whether targeting these molecules that are known to be aberrantly regulated in FD will have an effect on the clinical course.

Medical treatment is clearly indicated when the bone age in a patient with sexual precocity is advanced 2 years beyond the chronological age. While there is no consensus treatment for sexual precocity in MAS, both the aromatase inhibitor letrozole and the selective estrogen receptor modifier, tamoxifen, have shown efficacy.[141,142] A recent long-term follow-up study of the efficacy of letrozole in treating MAS-associated precocious puberty suggests that for several reasons it may be superior to tamoxifen.[143] Secondary central precocious puberty often ensues after several years of precocious puberty, which necessitates the addition of a gonadotropin analogue to the regimen. Treatment of sexual precocity in boys also involves the use of antiestrogenic drugs, such as letrozole to prevent bone age advancement and short stature, as well as an androgen receptor blocker, such as spironolactone or flutamide to prevent androgen-associated symptoms, such as acne and aggressive or age-inappropriate sexual behavior.[144]

Hyperthyroidism can almost always able to be controlled with antithyroidal medications; however, hyperthyroidism in MAS never resolves. One must consider whether lifetime antithyroidal medication is the best course or definitive treatment, such as surgery or radioactive iodine is a better choice at some stage.[124] Renal phosphate wasting with hypophosphatemia is typically treated in the same manner as is other disorders of phosphate wasting, such as X-linked hypophosphatemia. This entails phosphate supplementation and active vitamin D (calcitriol or alfacalcidiol). As is the case with this combination, patients are at risk for nephrocalcinosis and/or nephrolithiasis, for which subjects should be regularly monitored.

Unlike in cases of sporadic growth hormone excess, surgery is not the first line of treatment in patients with MAS and growth hormone excess for two reasons. First, the pituitary is always surrounded by FD bone, and the entire sphenoid sinus is typically replaced with FD in conjunction with a significantly FD-expanded sphenoid bone. As such the pituitary is usually inaccessible and the patient is inoperable. Second, even if the patient is deemed operable, the pituitary is usually diffusely involved with disease and only a total hypophysectomy would be curative. The diagnosis of Cushing's syndrome necessitates immediate attention. Bilateral adrenalectomy is curative, but at times the patients are too small and fragile to tolerate an operation of this magnitude. In these cases, medical treatment with metyrapone is possible to either palliate the patient until surgery can be performed, or in the hope that the cortisol excess will spontaneously resolve, as it has been shown to do in some cases.[125]

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CHAPTER

36

Genetic Disorders Caused by Mutations in the PTH/PTHrP Receptor, its Ligands, and Downstream Effector Molecules

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1 INTRODUCTION

The PTH/PTHrP receptor (also referred to as type I PTH/PTHrP receptor or PTH1R; gene name, *PTH1R*) is a G protein-coupled receptor that mediates the actions of two ligands, parathyroid hormone (PTH; gene name, *PTH*), and PTH-related peptide (PTHrP; also referred to as PTH-like peptide, PTHLP; gene name, *PTHLH*). The PTH1R stimulates at least two distinct second messenger pathways, cAMP/protein kinase A (PKA) and IP_3/Ca^{++}/PKC (for review see Ref. 1). It is most abundantly expressed in kidney, bone, and growth plates, and at lower levels in a large variety of other tissues. The critical role of PTH in endocrine regulation of mineral ion homeostasis had been explored for several decades through numerous studies in intact and parathyroidectomized animals.[2] Subsequent studies in mice with ablation of the genes encoding PTH[3] or GCM2,[4] a parathyroid-specific transcription factor, confirmed the importance of PTH for maintaining blood calcium levels within normal limits. PTHrP was first isolated from tumors that cause the humeral hypercalcemia of malignancy syndrome (for review see Ref. 1). Because its biological properties were largely indistinguishable from those of PTH, it was predicted that PTHrP acts through the same receptor than PTH, which turned out to be the case. However, through analysis of genetically manipulated mice, it became apparent that PTHrP has a particularly important role in the autocrine/paracrine regulation of chondrocyte growth and differentiation. Thus, animals that are "null" for *Pthlh* die in utero or shortly thereafter, and they show a profound acceleration of growth plate mineralization.[5] In contrast, mice overexpressing PTHrP under the control of a growth plate-specific promoter are viable, but show a severe delay in chondrocyte maturation which leads to impaired bone growth and elongation.[6] Consistent with these latter findings, homozygous ablation of the PTH1R leads to similar, albeit more severe developmental abnormalities.[7] The analysis of different, genetically manipulated animals thus provided important clues regarding the phenotypic abnormalities that were to be expected in humans with mutations in the PTH/PTHrP receptor, its ligands, or some of the downstream signaling and effector-proteins. Analysis of rare genetic disorders in humans has now provided additional insights into the molecules involved in these processes.

2 THE PTH/PTHrP RECEPTOR SYSTEM

2.1 Parathyroid Hormone (PTH)

Besides 1,25 dihydroxy vitamin D[1,25$(OH)_2$D], PTH is the most important endocrine regulator of extracellular calcium homeostasis in mammals.[1,8] PTH is almost exclusively expressed in the parathyroid glands; only lower protein and mRNA levels were identified in the hypothalamus and the thymus of *glial cells missing 2* (*gcm2*)-ablated mice.[4] Its synthesis and secretion by the parathyroid glands are dependent predominantly

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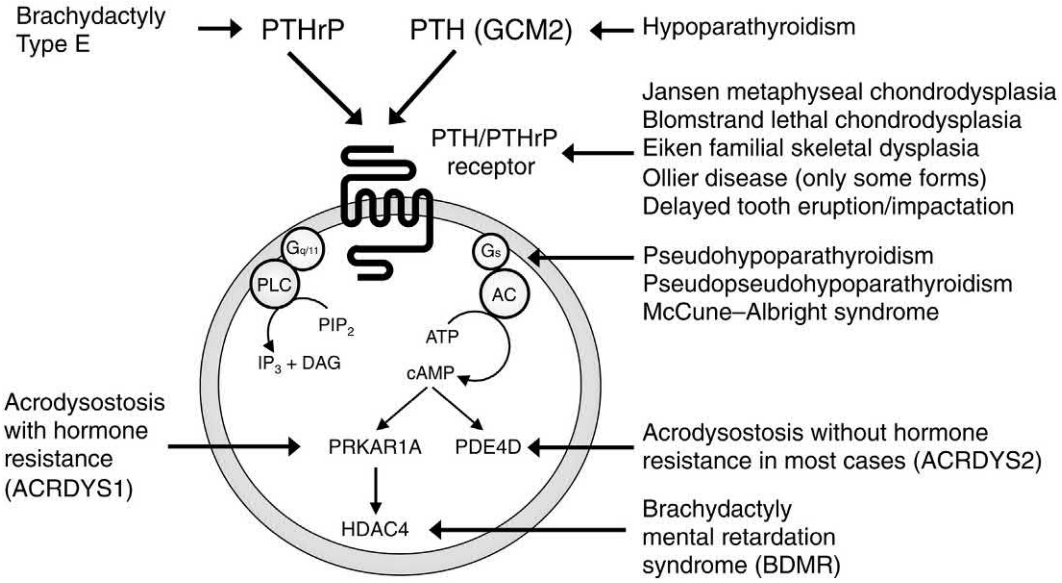

FIGURE 36.1 Human disorders and PTH1R-Gsα-cAMP-protein kinase A (PKA) signaling pathway. The PTH/PTHrP receptor (PTH1R) is abundantly expressed in kidney and bone where it mediates the PTH-dependent regulation of calcium and phosphate homeostasis. It is also expressed in numerous other tissues, particularly in the growth plate chondrocytes, where it mediates, following activation by PTHrP, the regulation of cellular proliferation and differentiation during development. The activated PTH1R stimulates two second messenger pathways, the cAMP/PKA and IP_3/Ca^{++}/PKC. Genetic diseases associated with defects in the cAMP/PKA pathway are indicated.

on the extracellular concentration of calcium,[9] monitored by the calcium-sensing receptor (CaSR),[10] and probably to a lesser extend by $1,25(OH)_2D$, phosphate, and FGF23.[9,11–14]

PTH acts primarily on kidney and bone, where it binds to cells expressing the PTH/PTHrP receptor and thereby initiates a series of processes that serve to maintain blood calcium and phosphate concentrations within narrow limits (Fig. 36.1). In kidney, the mRNA encoding the PTH/PTHrP receptor is expressed primarily in the convoluted and straight proximal tubules, the cortical portion of thick ascending limb and the distal convoluted renal tubules,[15–17] that is, in those renal segments which respond to PTH with an increase in cAMP accumulation.[18,19] PTH/PTHrP receptors are expressed on the basolateral and the brush-border membrane,[20] raising the possibility that PTH acts not only via the blood circulation from contraluminal side, but also, after glomerular filtration, from the luminal side.[20]

The most important PTH-mediated actions in the kidney affect the synthesis of $1,25(OH)_2D$from its precursor 25(OH)D, the excretion of phosphate, and the reabsorption of calcium. The stimulation of 1α-hydroxylase activity, an at least partially cAMP-dependent action,[21,22] is largely restricted to the proximal convoluted tubule. The resulting increase in $1,25(OH)_2D$ production enhances the absorption of calcium and phosphate from the intestine. The PTH-dependent inhibition of renal tubular phosphate reabsorption has been extensively documented in a variety of in vivo and in vitro studies (for reviews see Refs 23–26). To increase urinary phosphate excretion, PTH reduces the abundance of two type II sodium–phosphate co-transporters (NPT2a and NPT2c, also referred to as NaPi-IIa and NaPi-IIc) on the apical surface of proximal tubules.[23,27] In rodents, which can be readily studied, Npt2a is expressed in segments S1–S3, while Npt2c is expressed mainly in the S1 segment.[28] The acute PTH effects appear to be mediated in vivo prominently by cAMP/PKA-dependent mechanisms,[29] which is consistent with the severely diminished, PTH-stimulated phosphaturic response in patients with PHP, a group of related disorders that are caused by maternally inherited mutations in *GNAS*, the gene encoding Gsα, the alpha-subunit of the stimulatory G protein, and several different splice variants thereof.[30,31] However, studies with genetically engineered mice expressing a mutant PTH/PTHrP receptor that is deficient in signaling through Ca^{2+}/IP_3/PKC-dependent mechanisms have shown that this latter second messenger pathway contributes also to the PTH-mediated phosphaturic response.[32,33] The increase in urinary phosphate excretion stimulated by PTH is associated with increased internalization and subsequent lysosomal degradation of Npt2a, and similar mechanisms appear to apply to the regulation of Npt2c.[27,28,34]

PTH/PTHrP receptor protein expression has been demonstrated by immunohistochemical and immunoelectron microscopic analysis, and by functional studies, on both basolateral and luminal membranes in proximal tubular cells in vitro and in intact proximal tubules.[20,35,36] Apical receptors may be preferentially coupled to cAMP-independent signaling pathway,

while basolateral receptor activation initiates both cAMP-dependent and -independent effects.[37,38] Megalin, a multifunctional clearance receptor expressed on the apical surface of proximal tubular cells, has been shown to contribute to catabolism of PTH and it may antagonize PTH/PTHrP receptor activity.[39] In the distal convoluted tubule, PTH stimulates, possibly through second messengers other than cAMP, the reabsorption of calcium,[40,41] which is associated with an increased expression of the calcium channel TRPV5.[42]

Similar to its renal tubular effects, the PTH-dependent actions on bone are complex and difficult to study. As outlined later in more details, PTH can influence, either directly or indirectly, the proliferation and differentiation of several bone cell precursors. Furthermore, the effects resulting from PTH stimulation of mature osteoblasts appear to be different depending on the intensity and duration of the stimulus, the type of bone (trabecular versus cortical), and the hormonal impregnation of bone. As a result, the hormonal effects observed in vitro often fail to reflect the conditions in vivo. For example, PTH stimulates both bone formation and osteoclastic bone resorption; however, the continuous administration of PTH in vivo is thought to favor bone resorption over bone formation, whereas intermittent doses of the hormone results in net anabolic effects.[43,44] However, the transgenic expression of a constitutively active human PTH/PTHrP receptor under the control of the collagen type Ia1 promoter to target expression to osteoblasts,[45] or under the control of the DMP1 promoter to target expression to osteocytes,[46] leads to a dramatic increase in bone formation suggesting that continuous low-level receptor activation leads to similar bone changes as intermittent treatment with PTH.

2.2 Parathyroid Hormone-Related Peptide (PTHrP)

PTHrP was first discovered as the major cause of the humoral hypercalcemia of malignancy syndrome (for review see Ref. 1). Within its amino-terminal portion, PTHrP shares partial amino acid sequence homology with PTH, and as a result of these limited structural similarities, amino-terminal fragments of both peptides have largely indistinguishable biological properties, at least when tested in different in vitro systems. PTHrP and its mRNA are also found in large variety of fetal and adult tissues suggesting that this peptide has an important biological role throughout life. In fact, PTHrP has a prominent role in the regulation of chondrocyte proliferation and differentiation during the process of endochondral bone formation[7] and epithelial–mesenchymal interactions during organogenesis of certain epithelial organs, including skin, mammary gland, and teeth.[47–50]

2.3 The PTH/PTHrP receptor and its role in endochondral bone formation

PTH/PTHrP receptor belongs to the class B family of heptahelical G protein-coupled receptors (GPCR), which also comprises the receptors for secretin, calcitonin, glucagon, and several other peptide hormones; it binds PTH and PTHrP.[1] Similar to the widely expressed PTHrP, the mRNA encoding the PTH/PTHrP receptor is found in a large variety of fetal and adult tissues,[15,51] and at particularly abundant concentrations in proximal tubular cells, in osteoblasts, and in prehypertrophic chondrocytes of metaphyseal growth plate.[7]

Mice with ablation of both *Pthlh* alleles die during the perinatal period and show striking skeletal changes, which include domed skulls, short snouts and mandibles, and disproportionately short extremities, yet no obvious developmental defects in other organs. These skeletal changes are caused by a dramatic acceleration of chondrocyte differentiation that leads to premature growth plate mineralization. Heterozygous animals, lacking only one copy of the *Pthlh* gene, show normal growth and development, and are fertile, but develop, despite apparently normal calcium and phosphorus homeostasis, mild osteopenia later in life.[52] Growth-plate abnormalities that are, in many aspects, the opposite of those found in *Pthlh*-ablated mice are observed in animals that overexpress *PTHLH* under the control of the collagen α1(II) promoter.[6] Throughout life, these animals are smaller in size than their wild-type litter mates and they show a disproportionate foreshortening of limbs and tail, which is most likely due to a severe delay in chondrocyte differentiation and endochondral ossification. Thus, too little or too much PTHrP expression in the growth plate leads to short-limbed dwarfism, although through entirely different mechanisms.

From these and other studies, it is now well established that PTHrP facilitates the continuous proliferation of chondrocytes in the growth plate, and that it postpones their programmed differentiation into hypertrophic chondrocytes. Consistent with this role of PTHrP in endochondral bone formation, earlier in vitro studies had shown that PTH (used in these studies instead of PTHrP) affects chondrocyte maturation and activity.[7] More recent studies confirmed these findings by showing that PTH and PTHrP stimulate, presumably through cAMP-dependent mechanisms, the proliferation of fetal growth plate chondrocytes, inhibit the differentiation of these cells into hypertrophic chondrocytes, and stimulate the accumulation of cartilage-specific proteoglycans that are thought to act as inhibitors of mineralization.[53,54] In the absence of these cartilage-specific PTHrP effects, growth plates of homozygous *Pthlh* gene-ablated mice have a thinner layer of proliferating chondrocytes, while the layer of hypertrophic chondrocytes is relatively

normal in thickness, but somewhat disorganized. Taken together these findings suggested that the lack of PTHrP accelerates the normal differentiation process of growth plate chondrocytes, that is, resting and proliferating chondrocytes undergo fewer cycles of cell division and differentiate prematurely into hypertrophic cells, which then undergo apoptosis before being replaced by invading osteoblasts.

The phenotypic changes in mice which are "null" for either *Pthlh* or the *PTH1R* are similar, and current evidence indicates that the autocrine/paracrine actions of PTHrP within the growth plate are mediated through the PTH/PTHrP receptor.[7] Furthermore, mice missing either *Pthlh* or its receptor are resistant to the actions of Indian Hedgehog (Ihh), a developmentally important protein, which is most abundantly expressed in growth plate chondrocytes that are about to differentiate into hypertrophic cells. *Ihh* binds directly to *patched*, a membrane receptor, which interacts with smoothened, and thereby suppresses the constitutive activity of the latter protein.[55,56] The ectopic expression of *Ihh* in the chicken wing cartilage stimulates the production of PTHrP and thereby blocks the normal chondrocyte differentiation program; whether PTHrP represses, as part of a feedback loop, the expression of *Ihh* remains to be established. PTHrP and Ihh are thus critically important components of normal bone growth and elongation.[7] However, not all actions of PTHrP appear to be mediated through the PTH/PTHrP receptor, since the ablation of *Pthlh* or *Pth1r* leads to subtle, but distinctly different, abnormalities in early bone development.[57]

3 HUMAN DISORDERS CAUSED BY MUTATIONS IN THE PTH-PTHrP SIGNALING PATHWAY

3.1 *PTH*, *GCM2*, and *GNA11* Mutations

Only six *PTH* gene mutations have been identified as the cause of isolated hypoparathyroidism (IHP) in families with an autosomal dominant or recessive transmission.[58–63] Five of these mutations are located in the hormone's prepro leader segment and thus impair hormone synthesis or secretion. The more recently identified mutation, a homozygous (R25C) in the mature PTH(1–84) polypeptide reduces bioactivity of the secreted hormone.[63] Interestingly, depending on the assay used for evaluating patients carrying this mutation, plasma PTH levels were either low or elevated, thus leading to ambiguities regarding the underlying diagnosis, namely IHP or PHP1B. In this context, a novel homozygous mutation in the PTH1R (R186H) was hypothesized to result in an impaired interaction between PTH and PTH1R, thus postulating that this genetic defect leads to a PHP1B phenotype. However, the index case presented with severe hypocalcemia and hyperphosphatemia, yet normal PTH levels; she revealed no methylation changes at the *GNAS* locus. In contrast, two other family members, both the same homozygous R186H mutation in the PTH1R had laboratory findings consistent with PHP1B; however, no *GNAS* methylation studies were reported for these family members and no in vitro studies with the mutant PTH1R were conducted, thus raising doubts whether the identified mutation is indeed disease-causing.[64]

More frequent appear to be mutations in *GCM2* (*g*lial *c*ells *m*issing 2), the human homolog of the Drosphilia gene *Gcm*a, and a specific regulator of parathyroid gland development expressed exclusively in the parathyroid glands.[4] Studies of patients with IHP have shown that *GCM2* mutations can be associated with autosomal recessive and dominant forms of the disease.[62,65–70] Mutations in *GNA11*, the gene encoding Gα11, one of the signaling proteins at the CaSR, were shown to cause an autosomal dominant form of hypoparathyroidism,[71,72] which can be associated with short stature.[73,74] FAM111A mutations have been identified in patients with hypoparathyroidism associated with impaired skeletal development in the context of Kenny–Caffey syndrome type 2.[75,76]

3.2 *PTHLH* Mutations

Brachydactyly type E (BDE) is characterized by a general shortening of metacarpals and metatarsals and/or phalanges.[77,78] BDE can be isolated or it occurs as part of familial syndromes, such as *GNAS* haploinsufficiency[79]; furthermore, it is part of a syndrome that includes mental retardation besides brachydactyly (BMRD).[80] Within families the phenotype is usually variable, ranging from moderate shortening of individual metacarpals to a shortening of all bones in hands and/or feet.

Two groups have identified *PTHLH* haploinsufficiency as the cause of autosomal dominant BDE with short stature. *PTHLH* molecular defects causing BDE are heterogeneous and include (1) a balanced translocation, with breakpoints upstream of *PTHLH* on chromosome 12p11.2, that introduces a novel C-etys-1 site leading to a *cis*-regulated downregulation of *PTHLH* expression[81]; (2) a microdeletion on chromosome 12p encompassing *PTHLH*,[82] as well as smaller deletions within the *PTHLH*[83]; and (3) heterozygous missense, nonstop, and nonsense mutations.[82,84] In all studies, the mutations segregated with the disorder and were not found in control alleles. Thus, BDE with short stature is caused by mutations that lead to haploinsufficiency of *PTHLH*.

As observed in the other rare human syndromes associated with mutations in the *PTH1R* gene, BDE caused by *PTHLH* haploinsufficiency phenocopies important aspects of PTH1R functions, albeit with variable expressivities. Interestingly, a de novo duplication comprising

the *PTHLH* gene has been associated in one patient with symmetrical enchondromatosis.[85] These findings underscore the requirement for quantitatively normal levels of PTHrP expression for normal pre- and postnatal bone development and growth.[86]

3.3 *PTH1R* Mutations

3.3.1 *Jansen's Metaphyseal Chondrodysplasia*

Jansen's metaphyseal chondrodysplasia (JMC), first described in 1934,[87] is a rare autosomal dominant form of short limbed dwarfism associated with laboratory abnormalities that are typically observed only in patients with either primary hyperparathyroidism or with the humoral hypercalcemia of malignancy syndrome (reviewed in Refs. 88,89). These biochemical changes, that is, hypercalcemia, renal phosphate wasting, and increased urinary cAMP excretion, occur despite low or undetectable concentrations of PTH in the circulation and of PTHrP concentrations that are not elevated. Severe hypercalcemia, which is often asymptomatic, and hypophosphatemia had been noted in Jansen's first patient,[90] and in a subsequently described child with the same disorder.[91] It was not until the description of a third patient, however, that the association between the abnormalities in endochondral-bone formation and in mineral ion homeostasis was formally considered.[92] At that time the biochemical abnormalities could not be readily distinguished from those observed in primary hyperparathyroidism, but the surgical exploration of the patient revealed no obvious abnormalities of the parathyroid glands leading to the conclusion that the changes in mineral metabolism were caused by an undefined metabolic defect.[92] Most reported cases of JMC are sporadic, but the description of two unrelated affected females that gave birth to affected daughters[93–95] suggested an autosomal dominant mode of inheritance; this conclusion was subsequently confirmed for three families at the molecular level.[96–98]

At birth some patients with JMC have dysmorphic features, which can include high-skull vault, flattening of the nose and forehead, low-set ears, hypertelorism, high-arched palate, and micro- or retrognathia (for a review, see Ref. 88). Marked hypomineralization may also be observed suggesting hypophosphatasia.[99] Although, body length is within normal limits at birth, growth becomes increasingly abnormal, eventually leading to the development of short stature. Additional signs may include kyphoscoliosis with a bell shaped thorax and widened costochondral junctions, metaphyseal enlargement of the joints, waddling gait, prominent supraorbital ridges, and frontonasal hyperplasia. The legs are usually bowed and short, while the arms are relatively long.

Radiological studies have shown considerable, age-dependent differences in the osseous manifestations of JMC. In younger patients, severe metaphyseal changes, especially of the long bones, are present (Fig. 36.2). The metaphyses are enlarged and expanded, giving a club-like appearance to the ends of the long bones with a wide zone of irregular calcifications. Patches of partially calcified cartilage that protrude into the diaphyses are also present and appear relatively radiolucent. These findings, which are characteristically observed throughout early childhood, are similar to the lesions observed in rickets. However, distinct from the findings in rickets, metacarpal, and metatarsal bones are also involved.

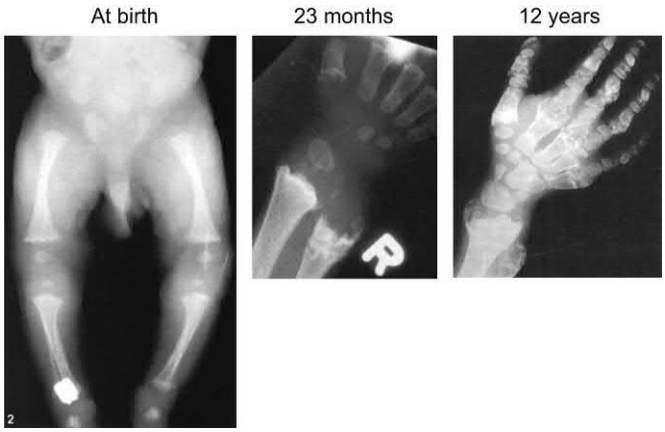

FIGURE 36.2 A patient with Jansen's disease and his radiological findings at birth, 23 months, and 12 years of age. *Source: From Silverthorn KG, Houston CS, Duncan BP. Murk Jansen's metaphyseal chondrodysplasia (JMC) with long-term followup.* Pediat. Radiol *1983;***17***:119–23 with permission.*[100]

Later in childhood, the changes are no longer reminiscent of rickets. Until the onset of puberty, almost all tubular bones show irregular patches of partially calcified cartilage that protrude into the diaphyses; the spine and vertebral bodies show no obvious abnormalities. After adolescence, the cartilaginous tissue in the metaphyses gradually disappears and turns into bone, leading to bulbous deformities (Fig. 36.2). The ends of most tubular bones remain expanded, deformed, and radiolucent, but a more normal trabecular pattern gradually emerges.[88]

In addition, sclerosis and thickening of the base of the skull and of the calvaria is noted in most cases. The former changes are thought to be the cause of cranial auditory and optical nerve compression, which has been observed later in life in some affected individuals. Loss of the normal cortical outline, areas of subperiosteal bone resorption, and generalized osteopenia are reminiscent of the changes seen in hyperparathyroidism. Furthermore, there is an increased in trabecular bone volume and a thinning of cortical bone.[89] The two only reports that investigated the histological changes in the growth plates, described a severe delay in endochondral ossification of the metaphyses, including a lack of the regular columnar arrangement of the maturing cartilage cells, a lack of excess osteoid (which is usually indicative

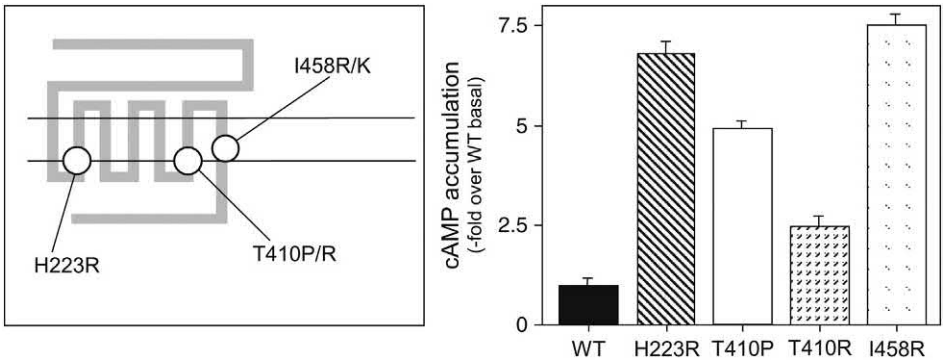

FIGURE 36.3 Schematic representation of the PTH/PTHrP receptor and basal, agonist-independent cAMP accumulation of wild type and mutant receptors. The approximate location of five different missense mutations that were identified in patients with Jansen's disease is indicated (left panel). Basal cAMP accumulation of COS-7 cells expressing wild type and mutant PTH/PTHrP receptors (H223R; T410P; T410R; I458R; right panel); note that the I458R and the I458K mutations have indistinguishable effects when tested in vitro.[99] *Source: Modified from Schipani E, Langman CB, Parfitt AM, Jensen GS, Kikuchi S, Kooh SW, Cole WG, Jüppner H. Constitutively activated receptors for parathyroid hormone and parathyroid hormone-related peptide in Jansen's metaphyseal chondrodysplasia.* New Eng J Med *1996;***335***:708–14.*[96]

of active rickets or osteomalacia), little or no vascularization of cartilage, and no evidence for osteitis fibrosa.[91,101] Tooth development and enamel formation appear normal in patients with JMC. Intelligence appears normal in all reported cases.

Most laboratory findings in JMC are reminiscent of those observed in patients with primary hyperparathyroidism or with the syndrome of humoral hypercalcemia of malignancy. In the newborn, blood phosphorus levels are typically at the lower end of the normal range, while alkaline phosphatase activity is almost invariably elevated. Hypercalcemia is usually absent at birth, but develops during the first months of life, and persists throughout life, but is more pronounced during infancy and childhood. Hypercalciuria is usually present and can be associated with an increased incidence of nephrocalcinosis.[102,103] $1,25(OH)_2D$ levels have been reported to be normal or at the upper end of the normal range. Serum alkaline phosphatase activity and osteocalcin concentration are elevated throughout life, indicating that osteoblast activity is increased; compatible with an increased osteoclastic activity, urinary hydroxyproline excretion is elevated.[104,105]

3.3.1.1 Jansen's Disease is Caused by Activating PTH/PTHrP Receptor Mutations

Because of the findings in the various genetically manipulated mice described previously, and because of the abundant expression of the PTH/PTHrP receptor in the three organs that are most obviously affected in JMC, that is, kidney, bone, and metaphyseal growth plates, activating receptor mutations were considered as a cause of this rare disease. Indeed, in several unrelated patients with this disorder, a heterozygous nucleotide exchange, which changes a histidine at position 223 to arginine, was identified at the border between the first intracellular loop and the second membrane-spanning domain of the PTH/PTHrP receptor.[106] In other patients, additional heterozygous nucleotide exchanges were identified which change either a threonine at position 410 to proline or arginine (sixth membrane-spanning domain), or isoleucine at position 458 (seventh membrane-spanning domain) to arginine[106] or to lysine[99] (Fig. 36.3 and Table 36.1). The mutated residues are predicted to be located at or close to the intracellular surface of the cell membrane and are strictly conserved in all mammalian members of this receptor family,[1] suggesting an important functional role for these three residues. With the exception of three families where parental transmission of the *PTH1R* mutation was documented,[96–98] each of the known mutations was excluded in the healthy parents and siblings, and could not be found in DNA from healthy controls and in different publically accessible databases. This suggests that JMC is usually caused by de novo mutations.

To test in vitro the functional consequences of the identified missense mutations in JMC, each of the four different nucleotide exchanges was introduced into the cDNA encoding the wild-type human PTH/PTHrP receptor.[122] COS-7 cells transiently expressing PTH/PTHrP receptors with either the H223R, the T410P, the T410R, or the I458R mutation showed significantly higher basal accumulation of cAMP, then cells expressing the wild-type PTH/PTHrP receptor (Fig. 36.3); likewise CHO cells expression either the I458R and the I458K mutant showed similar increases in basal cAMP production.[99] Cells expressing several of the mutant PTH/PTHrP receptors showed no evidence for increased basal accumulation of IP3, indicating that this signaling pathway is not constitutively activated.[105]

The T410R mutation seems to lead to a less severe form of JMC.[97] Patients carrying this mutation show only mild skeletal dysplasia with relatively normal stature, and serum calcium levels that are within the normal

TABLE 36.1 Mutations in the PTH/PTHrP Receptor Gene Associated With Different Human Disorders

Category	Location (exon NM_000316/recept or domain)	Nucleotide change	Amino acid change	Mutation type	Comment	Disease	References
LOF	STS markers 4–7 (10 kb comprising exon U3 to most of intron 1)			Deletion	Homozygous	Blomstrand	Unpublished, personal data
LOF	ex 3/E2	c.310C > T	p.R104X	Nonsense	Homozygous	Blomstrand	Hoogendam et al.[107]
LOF	ex 3/E3	c.331G > T	p.E111*	Nonsense	Heterozygous	PFE	Roth et al.[108]; Pilz et al.[108a]
LOF	ex 4/E3	c.356C > T	p.P119L	Missense	Heterozygous	PFE	Yamaguchi et al.[109]; Roth et al.[108]
Regulatory	ex 4/E3	c.362G > A	p.G121E	Missense	Heterozygous, enchondromas (not constitutional)	Enchondromatosis	Couvineau et al.[110]
Regulatory	ex 4/E3	c.364G > A	p.A122T	Missense	Heterozygous, enchondromas (not constitutional)	Enchondromatosis	Couvineau et al.[110]
LOF	ex 4/E3	c.395C > T	p.P132L	Missense	Homozygous	Blomstrand	Zhang et al.[111]; Karaplis et al.[112], Hoogendam et al.[107]
LOF	ex 4/E3	c.395C > T	p.P132L	Missense	Heterozygous	PFE	Yamaguchi et al.[109]
LOF	ex 4/E4	c.436C > T	R146*	Nonsense	Heterozygous	PFE	Roth et al.[108]; Pilz et al.[108a]
LOF	ex5/G	c.439C > T	p.R147C	Missense	Heterozygous	PFE	Yamaguchi et al.[109]
GOF (?)	ex5/G	c.448C > T	p.R150C (rs73067029)	Missense	Heterozygous	Enchondromatosis	Hopyan et al.[113]
LOF	ex5/G	c.463G > T	p.E155*	Missense	Heterozygous	PFE	Decker et al.[114]
LOF	ex6/M1	c.543+1G > A	p.?	Altered splicing	Heterozygous	PFE	Decker et al.[114]
LOF	ex6/M1	c.544-26 544-23del	p.E182Afs*38	Frame shift	Heterozygous	PFE	Risom et al.[115]
LOF	ex6/M1	c.557G > A	p.R186H	Missense	Homozygous	PHP1B	Guerreiro et al.[64]
LOF	ex 6/M2	c.572delA	p.Tyr191Serfs*14	Frameshift	Heterozygous	PFE	Frazier-Bowers et al.[116]
LOF	ex 7/M2	c.611T > A	p.Val204Glu	Missense	Heterozygous	PFE	Jelani et al.[117]
LOF	ex 7/M2	636dupT	Arg213*	Frameshift	Heterozygous	PFE	Roth et al.[108]; Pilz et al.[108a]
LOF	ex 8/M3	639-2A > C	p.?	Splice defect	Heterozygous	PFE	Roth et al.[108]; Pilz et al
GOF	ex 7/M2	c.668A > G	p.H223R	Missense	Heterozygous	Jansen	Schipani et al., [96,98,103,105,106]; Minagawa et al.[117a]
Regulatory	ex 7/M2	c.764G > A	p.R255H	Missense	Heterozygous	Enchondromatosis	Couvineau et al.[110]
LOF	ex 8/M3	c. 813dupT	A272Cfs*127	Duplication	Heterozygous	PFE	Roth et al.[108]; Pilz et al.[108a]

(*Continued*)

TABLE 36.1 Mutations in the PTH/PTHrP Receptor Gene Associated With Different Human Disorders (*cont.*)

Category	Location (exon NM_000316/recept or domain)	Nucleotide change	Amino acid change	Mutation type	Comment	Disease	References
LOF	ex 8/M3	c.892T > G	p.Trp298Gly	Missense	Heterozygous	PFE	Risom et al.[115]
LOF	ex 8/M3	c.947C > A	p.Ser316*	Nonsense	Heterozygous	PFE	Risom et al.[115]
LOF	ex 8/M3	c.989G > T	p.Gly330Val	Altered splicing	Heterozygous	PFE	Risom et al.[115]
LOF	ex 8/M3	c.996_ 997insC	p.Ala333Argfs*66	Frameshift	Heterozygous	PFE	Frazier-Bowers et al.[116]
LOF	ex 8/M3	c.1016G > A	Trp339X	Nonsense	Heterozygous	PFE	Roth et al.[108]; Pilz et al.[108a]
LOF	ex 9/M4	c.1049 + 27C > T	p.G350fsX351	Splice donor	Homozygous	Blomstrand	Hoogendam et al.[107]
LOF	ex10/El2	c.1050 − 3C > G	p.Cys351Serfs*133	Frameshift	Heterozygous	PFE	Decker et al.[114]
LOF	ex10/El2	c.1082G > A	p.Trp361*	Nonsense	Heterozygous	PFE	Risom et al.[115]
LOF	ex10/El2	c.1093delG	p.V365CfsX141	Frameshift	Heterozygous	Blomstrand	Karperien et al.[118]
LOF	ex 11/M5	c.1148G > A	p.L373_R383del	Novel splice acceptor site	Compound Heterozygous	Blomstrand	Jobert et al.[119]
LOF	ex 11/M5	c.1148G > A	p.L373_R383del	Novel acceptor site	Heterozygous	PFE	Yamaguchi et al.[109]
GOF	ex12/M6-7	c.1228A > C	p.T410P	Missense	Heterozygous	Jansen	Schipani et al.[96]
GOF	ex12/M6-7	c.1229C > G	p.T410R	Missense	Heterozygous	Jansen	Bastepe et al.[97]
LOF	ex12/M6-7	c.1348_1350del	p.Phe450del	Deletion	Heterozygous	PFE	Risom et al.[115]
LOF	ex 13/M7	c.1354 − 1G > A	p.Gly452_ Glu465de I	Deletion	Heterozygous	PFE	Frazier-Bowers et al.[119a]
GOF	ex 13/M7	c.1373T > G	p.I458R	Missense	Heterozygous	Jansen	Schipani et al.[105]
GOF	ex 13/M7	c.1373T > G	p.I458K	Missense	Heterozygous	Jansen	Savoldi et al.[99]
Regulatory	ex 14/T	c.1453C > T	p.R485X	Nonsense	Homozygous	Eiken	Duchatelet et al.,[120]

The description of the sequence variant is based on the nomenclature described by den Dunnen JT and Antonarakis SE.[121]
GOF, Gain of function; LOF, loss of function.

range. When tested in vitro, the degree of constitutive activity of the T410R mutant was significantly lower, when compared with the H223R, T410P, and I458R mutants, respectively.[97,122] This finding provided the first evidence of an obvious correlation between severity of phenotypical features and degree of constitutive PTH/PTHrP receptor activity in Jansen disease. Surprisingly, while the H223R mutation is typically associated with profound hypercalcemia despite low/normal PTH levels, a recent report indicated that overt hypercalcemia is not always encountered in JMC caused by the H223R mutation.[98]

3.3.2 *Blomstrand's Lethal Chondrodysplasia*

Blomstrand's lethal chondrodysplasia (BLC) is a recessive human disorder characterized by early lethality, advanced bone maturation and accelerated chondrocyte differentiation, and most likely severe abnormalities in mineral ion homeostasis. The first patient was described by Blomstrand and coworkers in 1985[123]; descriptions of several other patients followed.[118,124–129] The disorder was shown to occur in families of different ethnic backgrounds and appears to affect males and females equally. Most affected infants are born to consanguineous parents (only in one instance were unrelated parents reported to have two offspring that are both affected by Blomstrand's disease[126]), suggesting that BLC is an autosomal recessive disease. Infants with BLC are typically born prematurely and die shortly after birth. Birth weight, when corrected for gestational age, appears to be normal, but may be overestimated because most infants are hydroptic; also the placenta can be immature and edematous. Nasal, mandibular, and facial bones are hypoplastic; the base of the skull is short and narrow; the ears are low set; the thoracic cage is hypoplastic and narrow with short thick ribs and hypoplastic vertebrae. In contrast, the clavicles are relatively long and often abnormally shaped, the limbs are extremely short, and only the hands and feet are of relatively normal size and shape. Internal organs show no apparent structural or histological anomalies, but preductal aortic coarctation was observed in most published cases. The lungs are hypoplastic and the protruding eyes typically show cataracts. Defects in mammary gland and tooth development, previously overlooked, were demonstrated in two studied fetuses with BLC. In these fetuses, nipples were absent, and no subcutaneous ductal tissue could be identified by histochemical analysis. Tooth buds were present, but developing teeth were severely impacted within the surrounding alveolar bone, leading to distortions in their architecture and orientation.[130] Interestingly, heterozygous loss-of-function mutations in the *PTH1R* have been associated with autosomal dominant, isolated primary failure of tooth eruption (PFE)[109,114] (see Section 3.3.5).

Radiological studies of patients with BLC reveal pronounced hyperdensity of the entire skeleton and markedly advanced ossification (Fig. 36.4). As aforementioned, the long bones are extremely short and poorly modeled, show markedly increased density, and lack metaphyseal growth plates. Endochondral bone

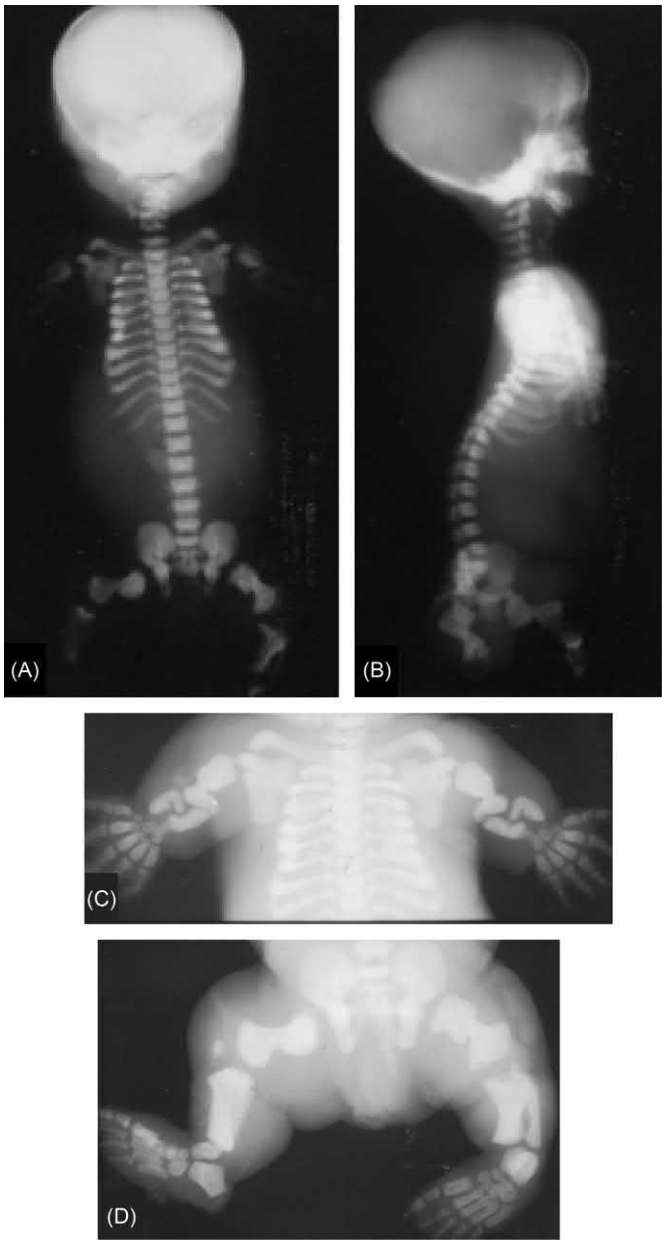

FIGURE 36.4 Radiological findings in two fetuses with Blomstrand's lethal chondrodysplasia (BLC) Anterio–posterior (A) and lateral (B) views of a male fetus at 26 weeks of gestation; upper (C) and lower (D) limbs of a female fetus with BLC at 33 weeks of gestation. Particularly striking is the dramatic acceleration of endochondral bone formation of all skeletal elements. No secondary ossification centers of ossification are seen in the long bones. The limbs are coarsely shaped and extremely short, while carpal and tarsal bones have a comparatively normal shape and size. Note also that the clavicles are relatively long, but show abnormal bending. *Source: From Loshkajian A, Roume J, Stanescu V, Delezoide AL, Stampf F, Maroteaux P. Familial Blomstrand chondrodysplasia with advanced skeletal maturation: further delineation.* Am J Med Genet *1997;***71***:283–8, with permission.*[126]

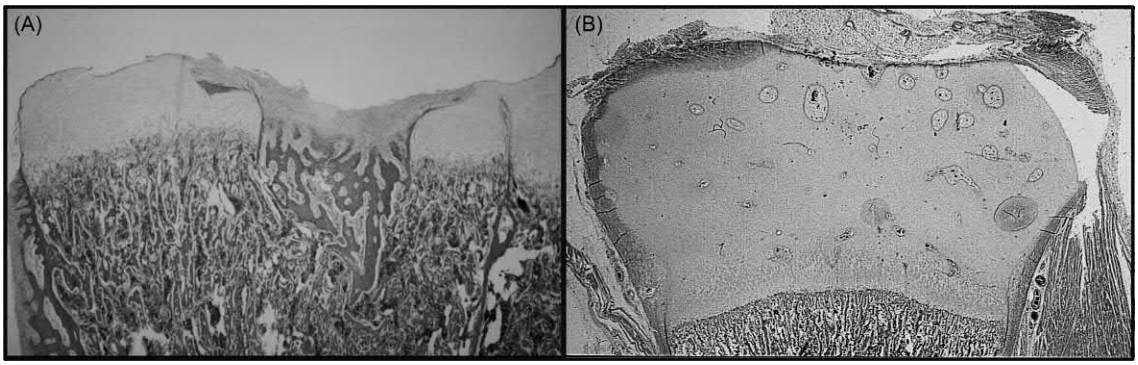

FIGURE 36.5 Section of the upper tibia end from a patient with BLC (A) and an age-matched control (B). Note the severely reduced size of the growth plate, the irregular boundary between the growth plate and the primary spongiosa, and the increased cortical bone thickness. *Source: From Loshkajian A, Roume J, Stanescu V, Delezoide AL, Stampf F, Maroteaux P. Familial Blomstrand chondrodysplasia with advanced skeletal maturation: further delineation.* Am J Med Genet *1997;**71**:283–8 with permission, and Anne-Lise Delezoide, personal collection.*[126]

formation is dramatically advanced, and is associated with a major reduction in epiphyseal resting cartilage preventing the development of epiphyseal ossification centers (Fig. 36.5). The zones of chondrocyte proliferation and of column formation are lacking, and the zone that normally comprises the layer of hypertrophic chondrocytes is poorly defined, narrow, and irregular.[131] Cortical bone is thickened, bone trabeculae are coarse with reduced diaphyseal marrow spaces. Capillary ingrowth, bone resorption, and bone formation are reported by some authors as being unaltered,[125] while others describe these bone remodeling events as deficient.[126]

3.3.2.1 Blomstrand's Disease is Caused by Inactivating PTH/PTHrP Receptor Mutations

Different defects in the PTH/PTHrP receptor gene have been described in genomic DNA from patients affected by BLC (Table 36.1). The first reported case, a product of nonconsanguineous parents, was shown to have two distinct abnormalities in the PTH/PTHrP receptor gene.[119] Through a nucleotide exchange in exon M5 of the maternal PTH/PTHrP receptor allele, a novel splice acceptor site was introduced which led to a mutant mRNA encoding an abnormal receptor that lacks a portion of the fifth membrane-spanning domain [amino acids 373–383; (Δ373–383)]. This receptor mutant fails, despite seemingly normal cell-surface expression, to respond to PTH or PTHrP with an accumulation of cAMP and inositol phosphate.[119] For yet unknown reasons, the paternal PTH/PTHrP receptor allele from this patient is very poorly expressed, suggesting an unidentified mutation in one of the different promoter regions or in a putative enhancer element.

A second patient with BLC, the product of a consanguineous marriage, was shown to have a nucleotide exchange that leads to a proline to leucine mutation at position 132 (P132L).[111,112] This residue in the amino-terminal, extracellular domain of the PTH/PTHrP receptor is invariant in all mammalian members of this family of GPCR, indicating that the identified mutation is likely to have significant functional consequences. Indeed, COS-7 cells expressing this mutant PTH/PTHrP receptor showed, despite apparently normal cell surface expression, dramatically impaired binding of radiolabeled PTH and PTHrP analogs, greatly reduced agonist-stimulated cAMP accumulation (Fig. 36.6), and showed no measurable inositol phosphate response. It is important to note, however, that cells expressing the P132L mutant receptor showed some agonist-induced second messenger response and showed little, but detectable specific binding of radiolabeled PTHrP. To date the P132L mutation has been identified in two additionnal patients affected with BLC.[107] Although, not definitive, haplotype analysis performed on the genomic DNA of these patients with the P132L mutation is consistent with an ancient founder effect.[107]

A homozygous deletion of G at position 1093 (from A of the ATG-translation initiation codon) (exon EL2) was identified in a third case of BLC.[118] This mutation led to a shift in the open reading frame, which resulted in a truncated protein that completely diverged from the wild-type receptor sequence after amino acid 364, and thus lacked transmembrane domains 5, 6, and 7, the connecting intra- and extracellular loops, and the cytoplasmic tail (Δ365–593).

As for the other cases of BLC, these findings provided a plausible explanation for the severe abnormalities in endochondral bone formation. The abnormalities in mammary gland and tooth development furthermore support the conclusion that the PTH/PTHrP receptor has in humans and mice identical roles in the development of these organs. Compatible with the role of PTH/PTHrP receptor and PTHrP in organogenesis, both were demonstrated to be expressed in the developing breast and tooth of human control fetuses.[130] It is also worth noting that abnormalities in skeletal development in the fetuses carrying the P132L mutation, which inactivates the PTH/PTHrP receptor incompletely, are less severe

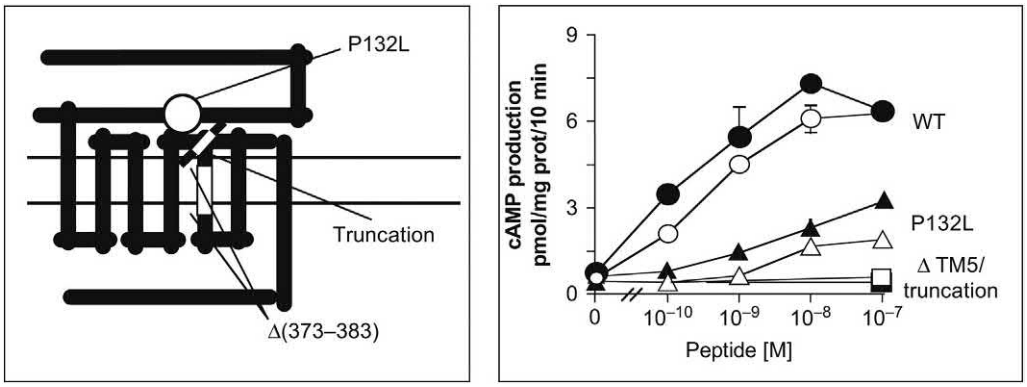

FIGURE 36.6 Schematic representation of the PTH/PTHrP receptor and functional evaluation of the wild type and mutant receptors in COS-7 cells. Approximate location of loss-of-function mutation identified in patients with Blomstrand's disease (left panel) and cAMP accumulation in response to PTH (closed symbols) or PTHrP (open symbols) by wild-type and mutant PTH/PTHrP receptors. *Source: Modified from Karperien MC, van der Harten HJ, van Schooten R, Farih-Sips H, den Hollander NS, Kneppers ALJ, Nijweide P, Papapoulos SE, Löwik CWGM. A frame-shift mutation in the type I parathyroid hormone/parathyroid hormone-related peptide receptor causing Blomstrand lethal osteochondrodysplasia.* J Clin Endocrinol Metab *1999;***84***:3713–20*[118]*; Zhang P, Jobert AS, Couvineau A, Silve C. A homozygous inactivating mutation in the parathyroid hormone/parathyroid hormone-related peptide receptor causing Blomstrand chondrodysplasia.* J Clin Endocrinol Metab *1998;***83***:3365–8.*[111]

than those observed in most cases, particularly with regard to the bones of the lower limbs.[118,124] This led to the proposal that two forms of BLC can be distinguished clinically and on the basis of the in vitro characteristics of the mutant PTH/PTHrP receptors.[131]

Two additional homozygous mutations in the PTH/PTHrP receptor have been identified in fetuses affected by BLC, which further document the molecular basis for the two forms of BLC[107] (Table 36.1). A homozygous point mutation causing a premature stop codon at position 104 (R104X) and therefore resulting in a truncated completely inactive protein has been identified in a case affected with the severe (type I) form. A homozygous nucleotide change (c.1049 + 27C > T, hg19) generating a novel splice site has been identified in a case affected with the less severe form (type II). This novel splice site, which results in an aberrant transcript with a premature stop codon after codon 350, was shown to be preferentially used in dermal fibroblasts, but the wild-type transcript remained expressed, albeit at low levels. Taken together the findings in patients with BLC suggested that this rare human disease is the equivalent of the mouse PTH/PTHrP receptor "knock-out."[132]

3.3.3 *Eiken Familial Skeletal Dysplasia*

In addition to BLC and JMC, PTH/PTHrP receptor gene mutations have been associated to two other diseases, Eiken familial skeletal dysplasia[120] and enchondromatosis (Ollier's disease).[113] Eiken familial skeletal dysplasia has been described in a single consanguineous family.[133] The disease is characterized by multiple epiphyseal dysplasia, with extremely retarded ossification, as well as by abnormal modeling of the bones in hands and feet, and abnormal persistence of cartilage in the pelvis and mild growth retardation. Serum calcium and phosphate levels have been normal in all the examined patients, serum PTH level was measured in only one patient and was found to be slightly elevated with normal 1,25$(OH)_2$D level. A homozygous mutation in the PTH/PTHrP receptor, R485X, that leads to the truncation of the last 108 amino-acids of the PTH/PTHrP receptor, was identified in all affected patients, but it was not found in DNA from healthy controls.[120] The functional properties of the mutant PTH/PTHrP receptor have not been characterized in vitro. However, based on the properties of a receptor mutant with deletion after amino acid 480,[134] it appears plausible that the truncated receptor has an unbalance between the different signaling pathways that are activated by PTH. Why the deletion of the carboxy-terminal tail of the PTH/PTHrP receptor results in a bone phenotype, but no obvious abnormality in the regulation of mineral ion homeostasis, remains unclear.

3.3.4 *Enchondromatosis (Ollier's Disease)*

Enchondromatosis is usually a nonfamilial disorder characterized by the presence of multiple enchondromas. It is characterized by an asymmetric distribution of the cartilaginous lesions, which can be extremely variable (in terms of size, number, location, evolution of enchondromas, age of onset and of diagnosis, requirement for surgery). Clinical problems caused by enchondromas include skeletal deformities, limb-length discrepancy, and the potential risk for malignant change to chondrosarcoma. The condition in which multiple enchondromatosis is associated with soft tissue hemangiomas is also referred to as Maffucci syndrome. The irregular distribution of the lesions in Ollier's disease strongly suggests that it is a disorder of endochondral bone formation that occurs due to a postzygotic somatic

mutation that results in mosaisism. A mutant PTH/PTHrP receptor (R150C) was found to be expressed in the enchondromas from two of six unrelated patients with enchondromatosis.[113] The mutation was found on one parental allele in one patient and his father, who presented with atypical mild skeletal dysplasia, but not with enchondromatosis. Consistent with a role of the PTH/PTHrP receptor in some forms of Ollier's disease novel heterozygous *PTH1R* mutations (either germline or somatic mutations in tumor tissue) have been found in several additional cases; no mutations were identified in *GNAS* or *PTHLH*.[110] As indicated previously, a de novo duplication comprising the *PTHLH* gene has been associated with symmetrical enchondromatosis.[85] However, neither the R150C mutation (26 tumors) nor any other mutation in the *PTH1R* gene (11 patients) could be identified in another study.[135] In fact, most of cases of Ollier's or the related Maffucci disease are now known to be caused by mutations in the isocitrate dehydrogenases IDH1 and IDH2. Somatic heterozygous mutations in IDH1 and less frequently in IDH2 were reported in 35 of 43 (81%) subjects with Ollier's disease, while 10 of 13 (77%) with Maffucci syndrome carried IDH1 (98%) or IDH2 (2%) mutations in their tumors. IDH1 and IDH2 mutations with evidence of intraneoplastic and somatic mosaicism were also identified in 87% of enchondromas (benign cartilage tumors), in 70% of spindle cell hemangiomas (benign vascular lesions), and in 40% of solitary central cartilaginous tumors.[136] IDH1 and IDH2 catalyze the oxidative decarboxylation of isocitrate to 2-oxoglutarate, which increases D-2-hydroxy-glutarate production and depletes the formation of α-ketoglutarate, a cofactor for the actions of Jumonju-domain containing proteins (JMJs) that are involved in the demethylation of histone arginine and lysine residues. Lack of α-ketoglutarate furthermore increases HIF-1α expression, which cause abnormal sensing of hypoxia within the growth plate cartilage, which may lead to an uncontrolled proliferation of chondrocytes. Taken together, these studies indicate heterogeneity in the molecular defects leading to enchondromatosis.

3.3.5 Delayed Tooth Eruption Due to PTH/PTHrP Receptor Mutations

PFE can be associated with several syndromes, but it is also observed as a nonsyndromic isolated autosomal-dominant condition with high penetrance and variable expressivity. Heterozygous *PTH1R* mutations were first identified in the individuals affected by PFE after the genetic locus had been determined through linkage analysis in four multiplex pedigrees.[114] Three distinct mutations were initially identified that are truncating the mature protein and are therefore expected to lead to a functionless receptor. These studies were subsequently confirmed and extended,[108,109,115–117,137] and indicate that haploinsufficiency of PTH1R is an underlying cause of nonsyndromic PFE. The PTH1R mutations responsible for PFE include nonsense, missense, splice-site mutations, and insertion-deletion mutations. Several missense mutations, including the P132L, were first identified in Blomstrand cases.[119] Tooth bud impaction has been described in Blomstrand's lethal osteochondrodysplasia,[130] thus supporting the important role of the PTH/PTHrP receptor in normal tooth development.

4 MUTATIONS IN GENES DOWNSTREAM OF THE PTH/PTHrP RECEPTOR

4.1 *GNAS* Mutations

Shortly after Aurbach and coworkers had discovered that kidney- and bone-derived tissues increase cAMP formation in response to PTH,[138–141] the same group revealed that patients with PHP and clinically obvious features of Albright's Hereditary Osteodystrophy (AHO) (now referred to as PHP-Ia) failed to respond to a PTH challenge with an increase in urinary cAMP excretion.[142] This observation linked the lack of PTH-induced phosphaturia, initially described by Albright and coworkers,[143] to this second messenger system. However, homogenates from the renal cortex of a PHP-Ia patient showed PTH-induced cAMP formation, leading Aurbach's group to the conclusion that this disorder is not caused by a mutation in the receptor for PTH (now referred to as the PTH/PTHrP receptor) or the effector enzyme that generates cAMP, that is, the adenylate cyclase.[144] In fact, it was the discovery that tissues from PHP-Ia patients show reduced G protein activity that provided first evidence for abnormal coupling between PTH-receptor and adenylate cyclase,[145–147] which subsequently led to the identification of heterozygous mutations in *GNAS*, the gene encoding the α-subunit of the heterotrimeric stimulatory G protein (Gsα).[30,31] However, it remained a conundrum why heterozygous Gsα mutations should lead to PTH-resistance, until Davies and Hughes revealed that the PTH-resistance in PHP-Ia patients becomes apparent only when the genetic defect is inherited maternally.[148]

It is now well established that Gsα, which is ubiquitously expressed, couples a large number of other GPCRs, including the PTH/PTHrP receptor, to the effector enzyme adenylyl cyclase that is required for receptor-stimulated intracellular cAMP generation and PKA activation. Gsα is derived from the *GNAS* locus, a complex imprinted genomic region located on chromosome 20q13, which encodes besides Gsα several other alternatively spliced transcripts[149] (detailed in Chapter 35). In some tissues, such as renal proximal

tubules (PTH target), thyroid or pituitary gland, the expression of Gsα is predominantly or exclusively monoallelic. In these tissues Gsα is derived mainly from the maternal allele, while expression from the paternal allele is silenced through as-of-yet unknown mechanisms.

Genetic and epigenetic defects in the *GNAS* complex locus cause, according to their allelic origin, heterogeneous diseases, that is, different forms of PHP, PPHP, and progressive osseous heteroplasia (POH), each with distinctive characteristics (detailed in Chapter 35). The clinical and biochemical distinction between these pathologies relies mainly, but not exclusively, on the presence or absence of the Albright hereditary osteodystrophy (AHO) and the presence of hormonal resistance, in particular PTH resistance. AHO is a distinctive constellation of variable developmental and skeletal defects (rounded face, short stocky appearance, BDE, early-onset obesity, heterotopic ossification, variable neurocognitive abnormalities) associated with Gsα haploinsufficiency. Obesity, initially thought to be part of the spectrum of AHO features, has now been shown to occur only upon maternal inheritance of *GNAS* mutations.[150] PHP is divided into type I (PHP1) and type II (PHP2). PHP1 is characterized by resistance to PTH in the proximal renal tubules resulting in little or no urinary cAMP excretion after injection of PTH. In PHP2, PTH injection causes a normal increase in urinary cAMP excretion, but no phosphaturic response. All forms of PHP1 and PPHP appear to be linked to genetic (PHP1A, PHP1C, and PPHP) and/or epigenetic (PHP1B) abnormalities at the *GNAS* complex locus. Patients who inherit Gsα mutations from their mother express both the AHO phenotype, as well as resistance to various hormones (PTH, TSH, calcitonin, GHRH, epinephrine, LH, and FSH) (for a review see Refs. 147,149–151) that stimulate the Gsα-cAMP-PKA pathway in their respective target tissues; this condition is referred to as PHP1A. PTH resistance is the most obvious endocrine deficiency that is almost always accompanied by hypocalcemia, hyperphosphatemia, and low or inappropriately normal levels of $1,25(OH)_2D$ despite elevated PTH levels (and absence of vitamin D deficiency or chronic kidney disease). Following injection of PTH, these patients exhibit a defect in the formation of cAMP in the renal proximal tubules and thus a reduced phosphaturic response and reduced $1,25(OH)_2D$ production. These findings were consistent with the loss-of-function mutations in Gsα, which is required for the formation of cAMP.

4.2 *PRKAR1A* Mutations

Acrodysostosis refers to a group of rare skeletal dysplasias that share common characteristic clinical and radiological features, including brachydactyly, facial dysostosis and nasal hypoplasia, and in some cases resistance to multiple hormones that mediate that actions through the cAMP/PKA pathway. Two forms of acrodysostosis are now recognized, type 1 (ACRDYS1, OMIM 101800) and type 2 (ACRYDS2, OMIM 614613), which are caused, respectively, by heterozygous mutations in the genes coding for the cAMP-dependent protein kinase type 1a regulatory subunit protein (PRKAR1A) and phosphodiesterase (PDE) 4D.[154,155] Using a candidate gene approach, a recurrent heterozygous missense mutation (c.1101C→T, p.Arg368X) was identified in three unrelated patients affected by ACRDYS1 in the gene *PRKAR1A*, which encodes the regulatory subunit of PKA.[41] Since then, 18 additional heterozygous missense *PRKAR1A* mutations have been documented in ACRDYS1 patients (Fig. 36.7A).[156–158] The first-described mutation, Arg368X, has been subsequently found in 10 additional patients, confirming that it is the most frequent recurrent *PRKAR1A* mutation in ACRDYS1. Each of the other 17 mutations was identified in a single patient, all sporadic cases.

As indicated previously, the most commonly used effector system downstream of cAMP is PKA. In the absence of cAMP, PKA exists as a tetramer in which two regulatory (R) subunits lock the two catalytic (C) subunits in an inactive state. Activation of the enzyme requires the release of the catalytic subunits, which is triggered by the sequential binding of cAMP molecules first to domain B, then to domain A of the R subunit.[159–161] Briefly, there are four different regulatory subunit isoforms; PRKAR1A and PRKAR2A are ubiquitously expressed, whereas PRKAR1B is found only in brain and testis (R1B) and PRKAR2B is found only in adrenal and adipose tissue (R2B). These regulatory subunits associate to two different catalytic subunits, the ubiquitous Ca and the brain specific Cb. The quantitatively and qualitatively predominant subunits are Ca and PRKAR1A; the Arg368X mutation results in deletion of the cAMP-binding site B of this regulatory subunit. This subunit represses constitutively the catalytic subunit, thereby impairing the PKA response to cAMP stimulation[41] (Fig. 36.7B). Subsequent functional characterization of PRKAR1A mutations causing acrodysostosis revealed that all mutations lead to a cAMP binding defect as the unique molecular mechanism for resistance of PKA activation.[162] This mechanism explains the hormonal resistance observed in ACRDYS1 patients and explains why their skeletal abnormalities resemble those observed in PHP-Ia/PPHP patients.

A knock-in of the recurrent R368X PRKAR1A mutation was recently developed in the mouse.[163] The heterozygous R368X KI mice presented the two main features of the ACRDYS1 phenotype in humans that is, renal proximal tubule resistance to PTH and chondrodysplasia. In addition the heterozygous R368X KI mice presented a striking and unexpected delay in endochondral

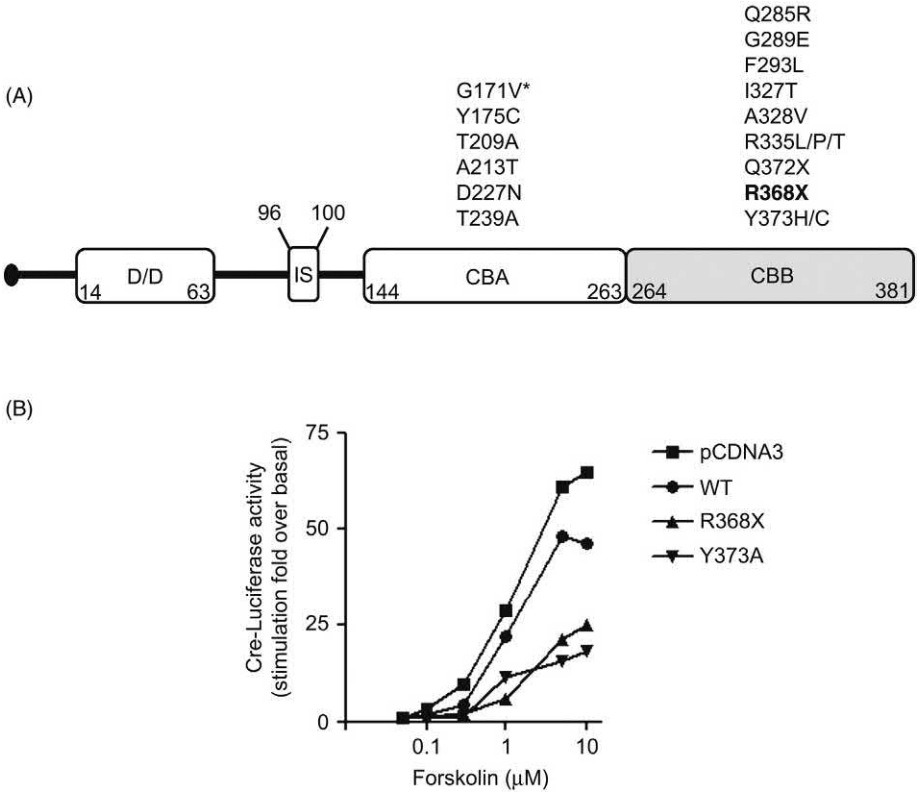

FIGURE 36.7 Panel A. Schematic representation of PRKAR1A protein indicating the functionally important domains and the position of the amino acid residues mutated in patients affected by acrodysostosis with hormonal resistance (ACRDYS1). DD, dimerization domain; IS, inhibitory site; CBA and CBB, cAMP-binding domain A and B. Panel B. PKA transcriptional activity in cells stimulated with various concentrations of forskolin in the presence of 1 mM isobutylmethylxanthine (IBMX) and as measured by the cAMP responsive element (CRE)–luciferase reporter assay. The results are expressed as -fold increase over basal values. After stimulation with forskolin, cells expression the R368X mutant or the Y373A mutant of PRKAR1A (used as a control) have significantly lower CRE–luciferase stimulation than cells expression the empty vector or wild-type PRKAR1A.

ossification, raising the possibility that PRKAR1A/PKA is a molecular switch at the crossroads of pathways orchestrating chondrocyte proliferation and differentiation. Of note, heterotopic ossifications, a hallmark of AHO and Gsα haploinsufficiency, are rarely observed in ACRDYS1.

4.3 *PDE4D* Mutations

Defects in *PDE4D*, first identified by exome sequencing, cause acrodysostosis without obvious evidence for hormonal resistance.[164–166] Recent work indicates that the ability of PDE4 to modulate signaling through GPCR-cAMP-PKA pathway depends on the cell type and stimulus intensity. Thus PDE4D mutations would be expected to impair some, but not all, responses, potentially explaining the presence of acrodysostosis without hormonal resistance in ACRDYS2.[166a] PDE4D belongs to the class IV of cAMP-specific phosphodiesterases that hydrolyzes cAMP. In humans, a single *PDE4D* gene gives rise, through differential splicing that modify the N-terminal portion of the protein to at least nine different isoforms.[167] All isoforms comprise the same C-terminal catalytic domain. Six isoforms, classified as "long" PDE4D variants, contain two conserved upstream regions (UCR1 and UCR2), while two short isoforms contain only the UCR2 region, and one "super-short" isoform of the enzyme contains only the catalytic domain. Little is known about the tissue-specific expression, function, and regulation of each of these isoforms in human tissues. To date, 32 heterozygous missense mutations in PDE4D have been identified in patients with acrodysostosis (ADOP4 or ACRDYS2)[156–158,168–170]. Interestingly, the PDE4D mutations are located in all three main functional domains (UCR1, UCR2, and catalytic), indicating that defects in all isoforms (long, short, and super-short) can putatively cause ACRDYS2. Based on the location of PDE4D mutations occurring in acrodysostosis without hormone resistance, it has been suggested that they make PDE4 insensitive to PKA regulation,[171] whereas mutations that would inactivate PDE4D (e.g., nonsense mutations, deletions, or mutations disrupting the catalytic site) have not been described. Recent findings indicate that PDE4D3 variants carrying ACRDYS2 mutations are more readily activated, over a wide range of intracellular

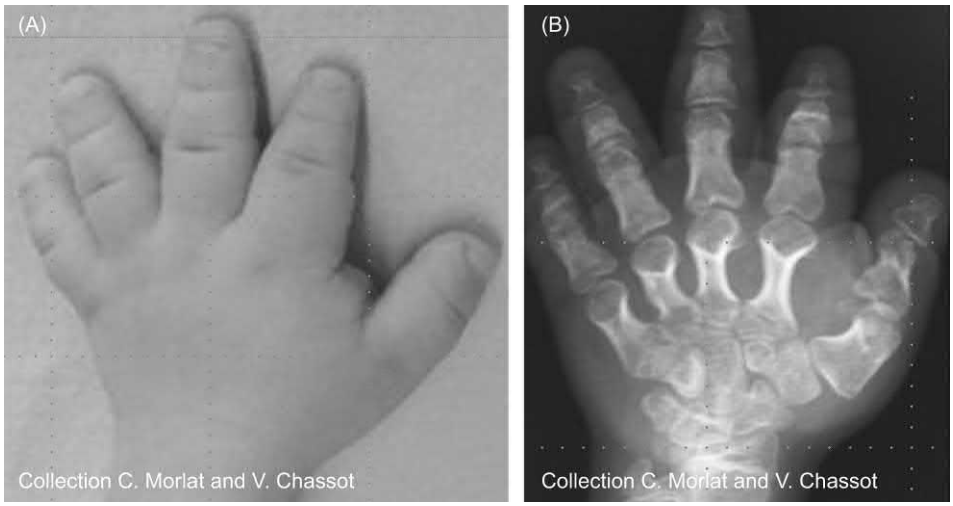

FIGURE 36.8 Brachydactyly in a patient with acrodysostosis and hormonal resistance (ACRDYS1 or ADOHR). Shown are a photograph (A) and radiograph (B) of one hand of a 13 years old patient. The patient presents a shortening of metacarpals, metatarsals and phalanges, characterizing brachydactyly type E (BDE). Note the shortening of all bones, and the bulky and stocky aspect of the affected bones.

cAMP concentrations, by PKA-induced phosphorylation than wild-type PDE4D3; furthermore, basal activity is enhanced.[172] These studies provide direct evidence for the conclusion that ACRDYS2 mutations result in increased hydrolytic activity of PDE4D. These findings may offer new perspectives into the selection of specific PDE inhibitors and possible therapeutic intervention for these patients. Another group, however, reported that PDE4D mutations causing acrodysostosis without hormone resistance resulted in impaired enzyme activity, and that the observed phenotype resulted from an overcompensatory increased expression of other PDE4 isoforms.[168]

Since the first reports, some clinical and radiological findings in acrodysostosis were noted to be similar to those observed PHP-Ia/PPHP syndromes,[154,155] however, distinct differences exist. In particular, although the different conditions present with stocky appearance and BDE, the brachydactyly in the GNAS-related disorders (Editor: GNAS should be in italics) is variable and usually less severe than in both forms of acrodysotosis. The skeletal phenotype in acrodysostosis is quite uniform and more severe than in PHP-Ia/PPHP (Fig. 36.8). In contrast to the severe skeletal dysplasia, resistance toward PTH and other hormones is less pronounced in ACRDYS1 than in PHP-Ia patient, and not present in patients affected by ACRDYS2.[41,157,166]

4.4 HDAC4 Mutations

Brachydactyly mental retardation syndrome (BDMR) presents with a range of features, including intellectual disabilities, developmental delays, behavioral abnormalities, sleep disturbance, craniofacial and skeletal abnormalities, comprising BDE, and autism spectrum disorder. BDMR had been associated with large deletions of 2q37. Clinical and molecular analysis of individuals with overlapping deletions involving 2q37.3 that refined this critical region led to sequencing of histone deacetylase 4 (*HDAC4*) as a candidate gene and identification of de novo mutations, including one intragenic deletion that probably disrupts normal splicing and one intragenic insertion thus causing a frameshift and premature stop codon in BDMR affected patients.[80] HDAC4 is a histone deacetylase that regulates genes important in bone, muscle, neurological, and cardiac development. Identification of *HDAC4* deletions or mutations in multiple subjects with BDMR, and the phenotype of *Hdac4*$^{(-/-)}$ mice indicate that haploinsufficiency of *HDAC4* results in BDMR syndrome.

5 CONCLUSIONS

Mutations in the genes encoding PTH, PTHrP, or the PTH/PTHrP receptor, and downstream signaling and effector proteins have been identified as the causes of rare inherited disorders. Identification of these mutations has provided important new insights into the regulation of bone and cartilage development, as well as mineral ion homeostasis.

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CHAPTER

37

Genetically Determined Disorders of Extracellular Calcium (Ca_o^{2+}) Sensing and Ca_o^{2+} Homeostasis

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1 INTRODUCTION

The extracellular calcium (Ca_o^{2+})-sensing receptor (CaSR), first cloned from bovine [1] and later from human parathyroid gland,[2] enables the maintenance of a nearly constant level of Ca_o^{2+} in the blood and other extracellular fluids. It does so by sensing even minute changes in Ca_o^{2+} from its normal level and then modulating the functions of key tissues, particularly the parathyroid glands and kidneys, so as to normalize Ca_o^{2+}.[3] The CaSR is expressed in numerous other cell and tissue types,[4] but its participation in systemic Ca_o^{2+} homeostasis relates principally to its physiological roles in the parathyroid and kidneys. In the parathyroid, the CaSR senses perturbations in Ca_o^{2+} of only a few percent, responding to hypocalcemia by increasing: (1) PTH secretion (within seconds), (2) expression of the PTH gene by virtue of increased stability of its mRNA (within hours to days),[5] and (3) parathyroid cellular proliferation (within days to weeks or longer).[3] In the kidney, CaSR-regulated responses to hypocalcemia include stimulation of Ca^{2+} reabsorption in the cortical thick ascending limb of Henle's loop [6] and, perhaps, activation of the 1-hydroxylation of 25-hydroxyvitamin D_3 to 1,25-dihydroxyvitamin D_3 in the proximal tubule.[6,7] Thus the hypocalcemia-evoked, CaSR-mediated stimulation of PTH release, which increases skeletal Ca_o^{2+} release, tubular reabsorption of Ca^{2+}, and formation of $1,25(OH)_2D_3$, coupled with the direct CaSR-mediated actions of hypocalcemia on tubular reabsorption of calcium and $1,25(OH)_2D_3$ formation, will elevate Ca_o^{2+} to its normal level. The CaSR also inhibits the urinary concentrating mechanism by inhibiting solute transport into the hypertonic medullary interstitium and antagonizing vasopressin action in the inner medullary collecting duct.[8] These actions of the CaSR on water homeostasis may indirectly impact calcium metabolism by increasing the volume of urine in which calcium is excreted in the setting of hypercalcemia and/or hypercalciuria, thereby potentially reducing the risk of renal stone formation.[6] While there has been some controversy as to the localization of the CaSR along the nephron, a recent study has reevaluated this issue using a variety of methodologies to detect CaSR protein and mRNA to clarify this issue.[9]

The CaSR also mediates the high Ca_o^{2+}-evoked stimulation of calcitonin (CT) secretion from the parafollicular (C)-cells of the thyroid gland,[10] which exerts a Ca_o^{2+}-lowering action by inhibiting osteoclastic bone resorption. While CT may not be physiologically important in humans, in mice it exerts a potent hypocalcemic action and protects against excessive bone loss during lactation.[11] The importance of the CaSR in the formation and turnover of bone has been somewhat controversial over the time since the receptor was cloned, but studies in mice with conditional knockout of the CaSR in osteoblasts within the past decade indicate key, nonredundant roles of the CaSR in this cell type.[12] Recent data also suggest that the skeleton can mediate PTH-independent, homeostatically relevant uptake or release of calcium during induced hyper- or hypocalcemia, respectively, actions that may involve the CaSR.[13] Nevertheless, in the conditions that will be discussed later in this chapter that impact the process of Ca_o^{2+} sensing, it is abnormalities in Ca_o^{2+}- and CaSR-regulated PTH secretion and renal calcium handling that are the most prominent in the

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associated phenotypes. Accordingly, these abnormalities will be the focus of the discussion that follows and potential perturbations in other CaSR-regulated processes will not be covered unless relevant.

Some understanding of the structure, function, and biosynthesis of the CaSR is important to appreciate the impact on the receptor of the naturally occurring inactivating and activating CaSR mutations that will be discussed later. The CaSR is a member of family C of the superfamily of G protein-coupled receptors (GPCRs),[14] which also includes the metabotropic glutamate receptors (mGluRs), the G protein-coupled receptors for γ-amino butyric acid (GABA), known as the $GABA_B$ receptors, pheromone receptors in rodents, some taste receptors, and GPRC6A. The last of these was cloned as an orphan receptor whose ligands and biological functions are still being unraveled, but its activators appear to include divalent cations, such Ca^{2+} and Mg^{2+}, osteocalcin, and others.[15] All of the family C GPCRs have large extracellular domains (ECD) that "sense" their respective ligands, which are all small molecules and/or atoms (e.g., glutamate, GABA, Ca^{2+}, and so on). In the CaSR, the 612 amino acid ECD has a cysteine-rich domain in its C-terminal region that is thought to serve as a link between the ECD and a 250 residue transmembrane domain (TMD), which contains 7 transmembrane helices, as well as 3 relatively short extracellular and 3 intracellular loops connecting the transmembrane helices (Fig. 37.1).[16] Following the TMD is an intracellular, 216 amino acid carboxy (C)-terminus containing several classes of consensus sequence motifs, such as those for phosphorylation by protein kinase A or C or interaction with various binding partners.[17]

The ECDs of several mGluRs have had their structures solved by X-ray crystallography and share a bilobed structure known as the Venus flytrap (VFT) domain.[18] Glutamate binds in the crevice between the two lobes, producing closure of the VFT and through as yet poorly understood mechanisms, bringing about changes in the conformations of the TMD and C-terminal tail that activate G proteins and a wide array of downstream-signaling pathways.[18] Molecular modeling strongly suggests that the ECD of the CaSR has a similar structure and that a key binding site for Ca^{2+} resides within the crevice between the two lobes of the ECD (Fig. 37.2).[19] Additional binding sites for Ca^{2+} may be present elsewhere in the ECD, close to or more distant from this first site, as well as in the TMD itself.[20–22] As with the mGluRs, binding of Ca^{2+} initiates intracellular signaling through multiple pathways (for review, see Refs. 22–24). Prominent among these are activation of phospholipase C and mitogen-activated protein kinases, both of which have been used to assess the function of naturally occurring mutants CaSRs.

During its biosynthesis, a signal peptide comprising the first 19 amino acids at the CaSR's amino-terminus directs the nascent receptor from the ribosome into the lumen of the endoplasmic reticulum (ER), after which the signal peptide is cleaved off.[1] In the ER, the CaSR dimerizes through two intercellular disulfide bonds involving cysteines 129 and 131 in the ECD.[25] Recent studies have identified arginine-rich, ER retention sequences within the proximal C-terminus of the CaSR that interact with binding partners and may regulate forward-trafficking of the receptor to the plasma membrane.[26] Because of the receptor's substantial retention in the ER, there is generally abundant CaSR present intracellularly as assessed by immunostaining. The initial N-linked core glycosylation of the ECD of the receptor occurs in the ER and facilitates forward trafficking to the Golgi apparatus, where the final maturation of the glycosylation to that present in the cell surface CaSR takes place.[27] From the Golgi, the CaSR traffics through the secretory pathway to the cell surface, where the dimeric, glycosylated CaSR has a molecular weight of about 320 kDa (160 kDa for each monomer), with the difference in molecular weight from that predicted from its amino acid sequence (~240 and ~120 kDa for the dimer and monomer, respectively) being accounted for by glycosylation.[28] Thus three species of CaSR monomer, mostly present in the receptor's predominantly dimeric forms, are present in varying amounts in CaSR-expressing cells—the nonglycosylated receptor (kDa ~120 kDa) as the initial biosynthetic product, the immature glycosylated receptor (kDa ~140) present in the ER and Golgi, and the mature fully glycosylated receptor on the cell surface (~160 kDa).[29] Recent studies have shown that intracellular CaSR can rapidly translocate to the cell surface upon activation of the receptor (so-called "agonist-driven insertional signaling" or ADIS), thereby ensuring continued, and, in fact, increased cell surface expression.[30] This ensures sustained downstream signaling despite the constant presence of the CaSR's principal ligand, Ca_o^{2+}, which might otherwise cause downregulation of the receptor.[30] As will be discussed later, specific mutations impact one or more aspects of the biosynthetic pathway, resulting in the presence of abnormal forms of the receptor or alterations in the relative quantities of the species of receptor just delineated.

The initial discovery of the CaSR and elucidation of its roles in calcium metabolism were followed shortly thereafter by the identification of several genetically determined disorders of extracellular calcium homeostasis. These result from inactivating mutations in the CaSR [familial hypocalciuric hypercalcemia (FHH, now called FHH1) and neonatal severe hyperparathyroidism (NSHPT)],[31] on the one hand, and activating mutations [autosomal dominant hypocalcemia/hypoparathyroidism (ADH, now called ADH1), which in some cases is accompanied by features of Bartter's syndrome],[32] on the other.[33] The discussion that follows will describe the

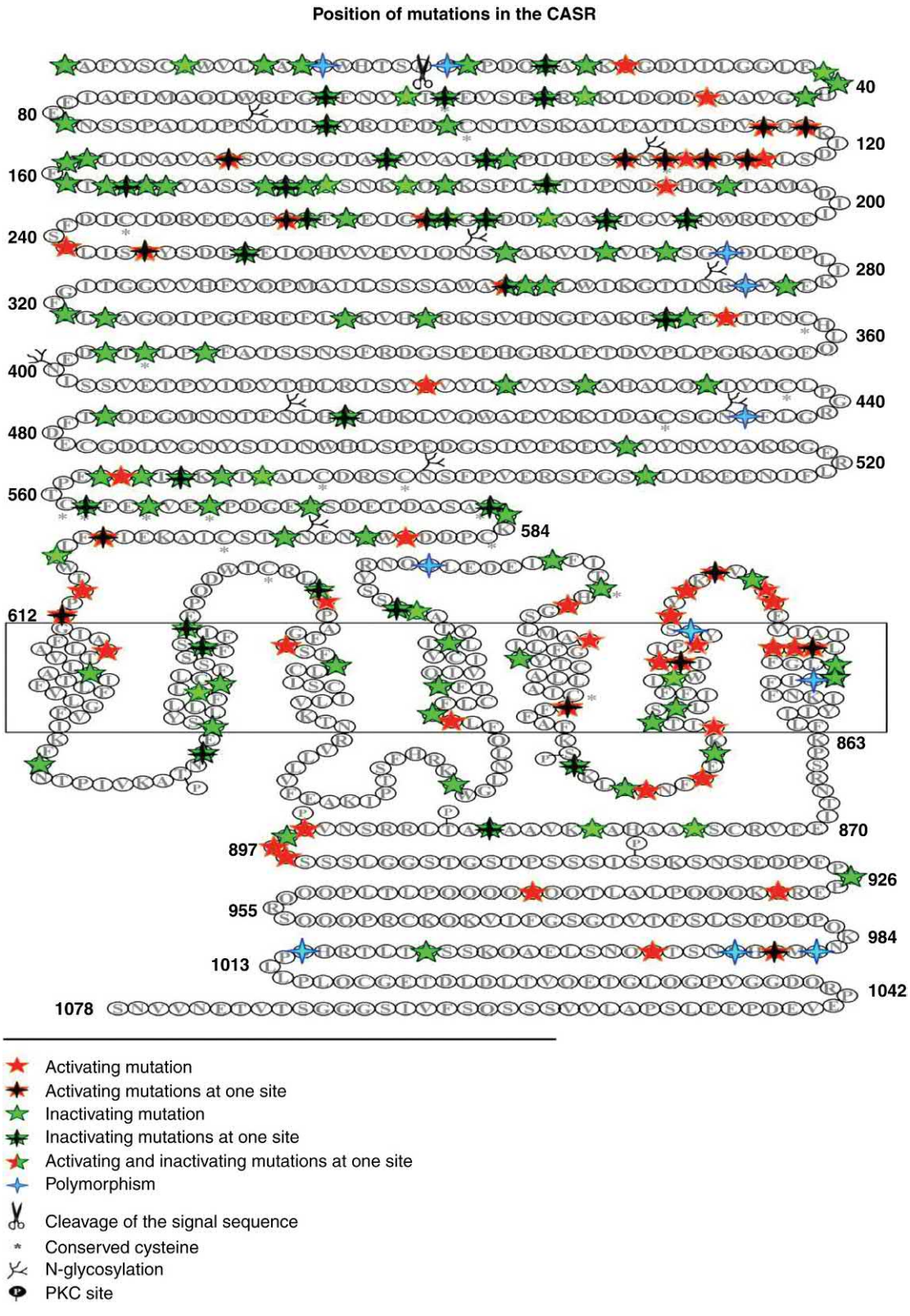

FIGURE 37.1 Topology of the CaSR, showing inactivating and activating mutations. The ECD of the receptor is shown at the top of the figure, while the seven membrane helices, three intra- and three extracellular loops and the intracellular C-terminal tail are shown. The key at the bottom describes the symbols used to illustrate the positions of inactivating and activating mutations, as well as polymorphisms. Note that several different inactivating or activating mutations can be present at the same residue, and that both an activating and an inactivating mutation can result from mutations of the same amino acid residue. *Source: Reproduced from Geoffrey Hendy, PhD and the CaSR database, with permission (http://www.casrdb.mcgill.ca/?Topic=CasrMutation&v=new).*

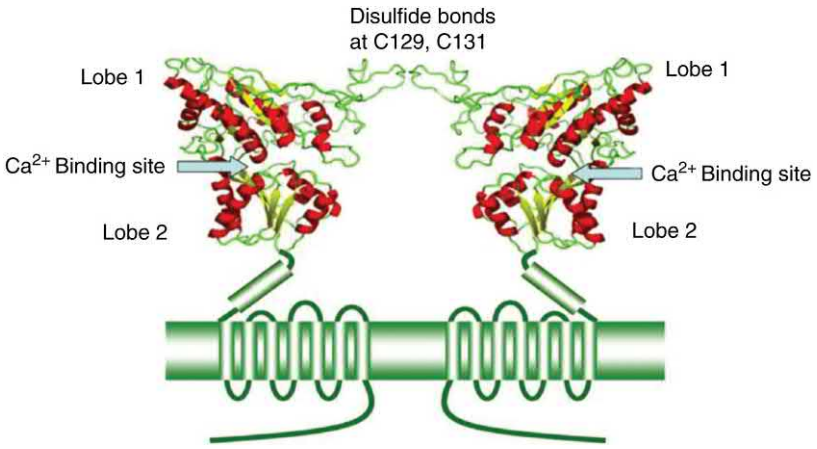

FIGURE 37.2 Model of the predicted structure of the dimeric CaSR. Depicted are the two extracellular domains (ECDs) of the dimeric CaSR. Alpha helices are shown in dark gray (*red* in the web version), and the approximate position of the two intermolecular disulfide bonds involving cysteines 129 and 131 are illustrated. Each monomer of the receptor's ECD assumes a bilobed Venus flytrap configuration with a binding site for calcium in the crevice between the two lobes of each monomeric ECD. The binding of calcium to this and perhaps other sites is thought to initiate closure of the VFT, followed by downstream intracellular signaling. Because it is dimeric, the CaSR is subject to dominant negative interactions between a normal and a mutant CaSR in a heterodimeric state. The seven membrane spanning helices of each CaSR monomer are shown in the lower portion of the figure. *Source: Reproduced from Huang et al.* J Biol Chem *2007;***282***:19000–10, with modifications according to the policies of the* Journal of Biological Chemistry.

characteristic features of these conditions as they present to the physician, geneticist, and/or pathologist and how they should be managed medically, including the use of genetic counseling, or, in some cases, surgically. Phenocopies of FHH and ADH caused by inactivating [34] or activating [35] antibodies to the CaSR, respectively, will be briefly alluded to as conditions that should be distinguished from the corresponding genetic syndromes. Moreover, the recent discoveries that FHH2 and ADH2 are caused by inactivating [36] and activating mutations,[36,37] respectively, of the *GNA11* gene, encoding the alpha subunit of the G protein, G_{11}, and that FHH3 is caused by inactivating mutations of the σ-1 subunit of the AP2 adaptor protein (AP2S1),[38–40] will be addressed as well.

2 CLINICAL AND GENETIC FEATURES OF FAMILIAL HYPOCALCIURIC HYPERCALCEMIA, INCLUDING FHH, TYPE 1 (FHH1) (OMIM #145980; ALTERNATIVE DESIGNATION, HHC1); FHH, TYPE 2 (FHH2) (OMIM #145981; ALTERNATIVE DESIGNATION, HHC2) AND FHH, TYPE 3 (FHH3) (OMIM #600740; ALTERNATIVE DESIGNATION, HHC3)

2.1 Clinical Features of FHH

In 1972, Foley et al. first described the characteristic clinical features of the apparently benign condition now known as FHH (initially called familial benign hypercalcemia).[41] Subsequently, Marx and coworkers, [42] as well as Heath and coworkers [43] in a series of papers published over the next 10–15 years reported many of the characteristic clinical and metabolic characteristics of this syndrome. Marx et al. applied the most commonly used term—FHH—to describe this syndrome. We now know that FHH can be caused by mutations in at least three genes, as noted earlier and described in detail later.

FHH1 presents most commonly as asymptomatic, mild to moderate hypercalcemia, ranging from only intermittently elevated serum calcium concentrations (and occasionally totally normal levels [44]) to values 1–2 mg/dL or more above the upper limit of normal, with an autosomal dominant pattern of inheritance and a high degree of penetrance.[42,43] As will be described in more detail later, FHH2 has a presentation similar to that of FHH1, while FHH3 has a degree of hypercalcemia that is, on average, more severe than that in FHH1 and FHH2, as well as additional phenotypic differences. The discussion that follows, particularly as it relates to studies carried out prior to the cloning of the CaSR, simply refers to the typical syndrome as FHH, while later studies are referred to in terms of their specific molecular cause (i.e., FHH1, 2, or 3), when known.

The hypercalcemia in FHH is accompanied by high normal or mildly elevated serum magnesium concentrations, mostly normal or sometimes mildly reduced serum phosphorus, normal plasma calcitonin concentrations, and normal PTH levels in about 80% of cases as measured by modern, two site assays.[43,45] Vitamin D

deficiency can cause an atypically high level of PTH in FHH1 and should be sought in this setting.[46] Serum levels of 1,25 dihydroxyvitamin D are generally within the normal range and lower than those typically found in primary hyperparathyroidism (PHPT).[45] Renal function is well preserved in FHH, and, despite their hypercalcemia, patients with typical FHH concentrate their urine more during a ~20 h water restriction than do those with PHPT and a comparable degree of hypercalcemia.[47] The excessively avid renal tubular reabsorption of calcium, which results from an abnormality in renal Ca^{2+} handling that is described later, is most commonly quantified as the ratio of renal calcium clearance to that of creatinine (usually referred to as the calcium to creatinine clearance ratio).[29,31] This parameter and how to calculate it are described in detail later in *Diagnostic aspects of FHH.* Bone mineral density in patients with FHH1 is equivalent to that of age-matched normal subjects and statistically higher than that in patients with PHPT.[48] Markers of bone turnover in FHH are normal or mildly elevated.[48]

Patients with typical FHH are asymptomatic, have no apparent long-term sequelae in most cases, and have a normal life expectancy.[42] Occasionally patients with FHH1, however, have recurrent bouts of pancreatitis.[49] Recent studies have readdressed the relationship of pancreatitis to FHH1. Of note in this regard, the CaSR is expressed in the pancreatic ducts, where alterations in its function could potentially be associated with pathology.[50] In one study, FHH1 patients who also harbored mutations in the *SPINK1* gene, which predisposes to the development of pancreatitis, appeared at increased risk of pancreatitis relative to those with *SPINK1* mutations alone.[51] In fact, mutations in the CaSR are now characterized by some investigators as one of six genes conferring an increased risk for pancreatitis.[52] However, it should be kept in mind that the development of pancreatitis in FHH1 is unusual and, when considered in the context of FHH1 and FHH as a whole, whether the incidence of this complication is higher in FHH than in the general population is not entirely clear.[53] Moreover, further studies are needed to define the potential role of specific CaSR mutations in predisposing of pancreatitis. There has been a report of idiopathic epilepsy linked to a missense mutation in the CaSR C-tail, which resulted in loss of an arginine in the arginine-rich ER retention sequence.[26] The proband was normocalcemic, however, and it was suggested that the mutation might produce CaSR dysfunction in the brain but not in parathyroid and kidney.

The identification of the genetic basis for most patients with FHH, namely heterozygous inactivating mutations in the CaSR [31] (for an updated list of mutations, see http://www.casrdb.mcgill.ca), has resulted in the recognition that there is a broader clinical presentation of FHH than previously recognized. Families with FHH1 occasionally have totally normal serum calcium concentrations. One such family was only identified because the homozygous offspring of a consanguineous union of two affected, normocalcemic family members was overtly hypercalcemic.[44] Rarely, therefore, as in this family, FHH1 presents in an autosomal recessive manner.[44] At the opposite end of the spectrum, some affected heterozygotes have serum calcium concentrations higher than the norm in this condition, as high as 13–14 mg/dL,[29] and, in some of these cases, PTH levels can be substantially elevated, suggesting a diagnosis of moderate to severe PHPT. The blurring of the distinction between FHH1 and PHPT has recently been further documented (vide infra). Genetic analysis of families with familial isolated hyperparathyroidism,[54] who were not clinically suspected of having FHH, revealed that CaSR mutations were present in 15%–20% of such families. In these families, therefore, FHH1 could not be reliably distinguished from PHPT on clinical and biochemical grounds alone, emphasizing the need for appropriate use of genetic testing. One family with clinical, biochemical, and pathological characteristics in the region of overlap between FHH1 and PHPT had overt hypercalciuria, rather than hypocalciuria, in some affected family members.[43] In this family, several affected individuals achieved long-term remission of their hypercalcemia after removal of one or more adenomatous parathyroid glands. Additional reports [55,56] have described patients presenting with renal calculi and/or the typical biochemical and clinical picture of sporadic PHPT, but who turned out to have CaSR mutations. In one study parathyroid exploration revealed a single adenoma in four such patients, three of whom became normocalcemic postoperatively,[56] while, in another study, parathyroid adenomas or hyperplasia were found at surgery in affected family members.[55] It is not currently known whether the occurrence of FHH1 and a parathyroid adenoma in the same patient is coincidental or whether the presence of an inactivating CaSR mutation predisposes in some way to the development of one or more adenomas. One study found that those individuals with mutations in the CaSR's TMD or C-tail were more likely to achieve normocalcemia following parathyroid surgery than were those whose mutations were in the ECD.[56] The favorable outcome described in these reports, at least in some patients, contrasts with the previously described results of parathyroid surgery in the majority of patients with FHH,[43] including patients with familial isolated hyperparathyroidism caused by CaSR mutations.[54] Namely, anything less than total parathyroidectomy (usually undertaken in the setting of a misdiagnosis of PHPT) was usually followed by prompt recurrence of PTH-dependent hypercalcemia, and, therefore, was of little clinical utility in asymptomatic patients with FHH. In symptomatic patients with FHH, however, it will be important to define further

whether clinical benefit will ensue from parathyroid surgery.

Two additional presentations of heterozygous inactivating mutations of the CaSR that have been uncovered in infants and children following the widespread application of mutational screening of the CaSR are NSHPT and neonatal hyperparathyroidism (NHPT).[57] The clinical, biochemical, and genetic features of NSHPT, which usually results from the presence of homozygous or compound heterozygous inactivating CaSR mutations, are described in detail later (see Section 3). NHPT and NSHPT, resulting from heterozygous CaSR mutations, are part of a spectrum in clinical and biochemical severity between typical FHH1 and more severe hyperparathyroidism in the neonatal period. The term NHPT has been applied to cases with nonlife-threatening elevations in serum calcium concentrations to ~11–12 mg/dL accompanied by marked elevations in serum PTH and hyperparathyroid bone disease.[57] These cases tend to revert over time to a phenotype similar to that of classical FHH1 with or without removal of hyperfunctioning parathyroid tissue. Some patients with heterozygous CaSR mutations may merit the diagnosis of NSHPT on the basis of more severe hypercalcemia and bone disease than seen in NHPT, but their serum calcium concentrations are generally lower (<~15 mg/dL) than that of most infants with homozygous CaSR mutations, which not infrequently exceed 20 mg/dL. In some cases, gestation of a fetus with FHH in a normal mother may excessively stimulate the fetal parathyroid glands because they are exposed to the normal maternal calcium concentration, which is sensed as in "hypocalcemic" by the abnormal parathyroid glands of the fetus.[58] The broad range of phenotypes resulting from heterozygous inactivating mutations of the CaSR is shown in Table 37.1.

TABLE 37.1 Phenotypes Associated With Inactivating CaSR Mutations

Heterozygous mutations
Typical FHH with hypercalcemia ranging from high normal to moderate
FHH with unusually severe hypercalcemia (e.g., dominant negative mutation)
NSHPT (serum calcium generally < 15 mg/dL)
NHPT
Familial isolated or sporadic PHPT
Homozygous or compound heterozygous mutations
NSHPT
Asymptomatic hypercalcemia in children or adults
PHPT in adulthood
Autosomal recessive hypercalcemia

NHPT, Neonatal hyperparathyroidism; NSHPT, neonatal severe hyperparathyroidism; PHPT, primary hyperparathyroidism.

By virtue of its typically benign clinical features, relatively little is known of the true prevalence of FHH. However, some increase in apparent prevalence has been reported with the advent of routine biochemical testing of blood chemistries, including calcium, as part of the investigation of other clinical conditions. One study from the West of Scotland estimated a prevalence of about 1 in 78,000.[59] At the present time, there is insufficient data to draw conclusions on racial predisposition, although FHH is relatively rarely reported in African Americans.[60] The prevalence of PHPT is considerably higher than that of FHH but varies geographically and in different reports,[61,62] with a prevalence for PHPT as high as 2%–3% in older members of Scandinavian populations, while in the United States the incidence in all comers in Rochester, MN is ~1:10,000. Therefore, while only a minority of cases of PHPT have biochemical features resembling FHH (e.g., about 10% of the former have relative hypocalciuria), the absolute number of PHPT cases whose biochemical features could potentially be confused with FHH may be similar to or substantially greater than the absolute number of FHH cases. Of note, in the study described earlier from Germany in whom inactivating CaSR mutations were sought in 139 patients with a provisional diagnosis of PHPT, in addition to the 4 patients with CaSR mutations, who presented with clinical and biochemical findings typical of PHPT, 4 others were identified with CaSR mutations in whom the diagnosis of FHH1 became clear in retrospect from their biochemical phenotype.[56] Thus, if the results of this study are confirmed, inactivating mutations in the CaSR may be more common than previously recognized, although earlier studies failed to identify CaSR mutations in more than 50 parathyroid adenomas.[63]

With regard to the clinical features of FHH2 and FHH3 and how they compare to those of FHH1, the very limited number of FHH2 cases that have been studied have not suggested any obvious differences in their biochemical findings with respect to calcium homeostasis.[36,37] With the identification of additional FHH2 kindreds, it will be of interest to determine whether there are phenotypic features resulting from heterozygous inactivation of the *GNA11* gene in tissues other than parathyroid and kidney.

Based on the features of the first family that was shown to be linked to the long arm of chromosome19, which we now know to encode the *AP2S1* gene, this form of the condition (FHH3) was called the *Oklahoma variant* (FBHOk).[64] Patients in this family exhibited hypophosphatemia, a tendency to an age-dependent rise in serum PTH to frankly elevated levels, and the presence of the bone disease—osteomalacia (defective mineralization of bone)—in some family members.[65] In a second family with FHH3 identified prior to the elucidation of the molecular basis for FHH3, however, these features,

which are atypical for the forms of FHH linked to chromosomes 3q or 19p, were less apparent.[66] However, two recent studies by Vargas-Poussou et al. [40] and Hannan et al. [67] in a combined total of 36 probands, in which FHH3 was documented based on the presence of AP2S1 mutations, have shown clear phenotypic and biochemical differences between patients with FHH types 1 and 3. Patients with APS2S1 mutations had more severe hypercalcemia (averaging around 3 mM or 12 mg/dL, but as high as ~3.4 mM in some cases), elevated levels of serum magnesium, and more marked hypocalciuria, as well as cognitive impairment and short stature in a substantial minority of cases.[40] A specific mutation (R15L) causing FHH3 exhibited the highest level of serum calcium concentration.[67] On average, all patients with FHH3 had higher plasma calcium concentrations in comparison to patients with FHH1, despite having similar PTH concentrations and rates of urinary calcium excretion. Renal tubular calcium reabsorption was also higher in patients with FHH3 than in those with FHH1. Bone mineral density was reduced in a small number of patients studied to date.[40,67]

2.2 Genetics of FHH

The three genetic forms of FHH have been indexed in the Online Mendelian Inheritance In Man (OMIM) database (http://www.ncbi.nlm.nih.gov/omim/145980). FHH1 has been indexed as MIM #145980 based on evidence that the phenotype attributed to this condition in many but not all cases is caused by a mutation in the gene encoding the calcium-sensing receptor (*CASR*, note that the designation of the gene differs from the common designation of the receptor protein as CaSR). Linkage analysis has shown that the genetic locus of FHH1 is on the long arm of chromosome 3, with an assigned locus of 3q13.3-21.[68] However, as noted previously, FHH is not always linked to chromosome 3q. Notably, a family, with clinical features similar to FHH showed linkage to the short arm of chromosome 19 (19p13.3, MIM #145981), and this form of FHH was designated FHH2.[69] This genetic form of FHH results from loss-of-function mutations in *GNA11*, which encodes $G\alpha_{11}$, a key G protein involved in CaSR signaling via the phospholipase C pathway (PLC).[36] Finally, the third genetically distinct form of FHH (designated as FHH3) is linked to the long arm of the same chromosome (19q13, MIM #600740) [65,66] and is caused by loss-of-function mutations of the *AP2S1* gene, which encodes the σ-1 subunit of the adaptor-related protein complex 2.[38] This complex is considered important for clathrin-mediated endocytosis of plasma membrane proteins, such as the CaSR, and inactivating mutations of the σ-2 subunit in FHH3 impair both CaSR-mediated signaling and endocytosis of the CaSR in heterologous expression systems.[38] Recent data from Vargas-Poussou indicate that FHH1 accounts for about 60% of cases thought to have FHH on clinical grounds, *AP2S1* mutations account for another 9% of these cases, and about 31% are of unknown genetic basis.[40] Only two families with FHH2 are known at present, [36,70] and this form of FHH appears substantially less common than FHH1and FHH3. It is entirely possible; therefore, that in the 31% of cases without an identifiable molecular cause of their FHH in this study, additional, genetically distinct forms of FHH exist that are caused by mutations in yet-to-be identified genes involved in Ca_o^{2+} sensing.

2.3 Molecular Genetics

At the time this chapter was updated for this edition, at least 208 inactivating mutations, as well as 11 polymorphisms, have been described in the coding region or splice sites of the *CASR* gene [comprising at least 170 missense (82%), 21 nonsense (10%), 13 insertion and/or deletion mutations (6%) (10 producing a frameshift and resultant stop codon), and 3 splice site mutations (<2%)] [see *CASR* gene mutation database (http://www.casrdb.mcgill.ca)] related to FHH and NSHPT (MIM #239200) either in families or as de novo disease (Fig. 37.1). [Mass sequencing efforts have identified a substantial number of additional, novel mutations in the CaSR, although the lack of clinical correlation with these mutations make them of uncertain significance, i.e., are these pathogenic or benign polymorphisms. Many of these can be accessed at https://www.google.com/url?q=http://cancer.sanger.ac.uk/cosmic/gene/overview%3Fln%3DCASR&sa=U&ved=0ahUKEwiLnOvihKDMAhWGYiYKHc7JCroQFggNMAA&sig2=eufN9d9STbUxrqij1QH1Iw&usg=AFQjCNGiDvlvWq8m-10si8eMLEGo_zelJw.] About half of the mutations in the CaSR causing FHH1 are in the first half of the ECD, a quarter in the second half of the ECD, a fifth in the TMDs, including the intracellular and extracellular loops, and less than 10% in the C-tail. The functional implications and genotype–phenotype correlations of these CaSR mutations are discussed in detail later (see Section 2.5). While not associated with overt clinical pathology, particular haplotypes of the polymorphisms in the CaSR's C-tail may contribute to variations in the serum calcium concentrations within the normal range,[71] presumably owing to subtle alterations in the receptor's function. In addition, a study of CaSR polymorphisms in renal stone-forming patients suggested that the 990G variant could influence renal CaSR activation and calcium excretion.[72] The role of CaSR polymorphisms in a variety of disease states is an active area of investigation.

Three inactivating mutations have been described in the *GNA11* gene in FHH2, T54M, L135GQ, and I200del. All of the mutations in AP2S1 causing FHH3 are located in codon 15, R15C, R15H, and R151L.[40,67] None of the

other potential missense mutations at this site have been observed, which has been interpreted as evidence for codon bias, perhaps because these other mutations are toxic when expressed in vivo or in vitro.[67] A description of the functional properties of these FHH1, FHH2, and FHH3 mutations when expressed in heterologous cell systems and mechanisms by which these mutations may disrupt the function of the normal gene are described later in Section 2.5.

2.4 Animal Models

Shortly after the cloning of the CaSR, mouse models of FHH and NSHPT were developed utilizing heterozygous or homozygous targeted inactivation of exon 5 of the CaSR, which is within the ECD of the receptor.[73] Mice with heterozygous "knockout" of the CaSR carried out in this manner have mild elevations in serum calcium concentrations and PTH compared to wild-type mice but are otherwise normal. These heterozygous mice replicate, therefore, the clinical and biochemical features of FHH. Mice with homozygous knockout of exon 5 of the CaSR, on the other hand, have much more severe hypercalcemia, marked elevations in serum PTH and parathyroid hyperplasia, as well as severe bone disease with demineralization and fractures.[73] The homozygous mice grow much more slowly than the wild type or heterozygous knockout mice and die within the first few weeks of life. Thus this phenotype is similar to that of NSHPT, as described in the next section of the chapter.

After these mouse models were created, it was discovered that an alternatively spliced, truncated form of the CaSR, which has in-frame deletion of the part of the ECD encoded by exon 5 of the *CASR* gene, is expressed in a variety of tissues.[74] It is thought to have biological activity and, therefore, to have the potential for "rescuing" the consequences of losing the full length CaSR in tissues participating in calcium homeostasis, such as bone and, to a lesser extent, parathyroid, kidney and C-cell. It has been difficult, however, to express the exon 5-less receptor on the cell surface of heterologous cell systems and to show that it possesses biological activity. Moreover, mice homozygous for knockout of exon 5 of the CaSR that have been "rescued" by homozygous deletion of the *PTH* gene from the severe hyperparathyroidism of mice with homozygous knockout of exon 5 alone, exhibit marked impairment of both high calcium-stimulated calcitonin secretion and renal calcium excretion,[10] suggesting the importance of the full length CaSR for these biological functions. Evidence in support of the key roles of the CaSR in cartilage and bone has been obtained using mice with tissue-specific knockout of exon 7 of the CaSR, encoding the CaSR's TMD and C-tail, in chondrocytes and osteoblasts, respectively.[12] Mice with heterozygous or homozygous global knockout of exon 7 have phenotypes faithfully reproducing these of FHH and NSHPT, respectively.[12] Studies are actively being pursued assessing the impact of the conditional knockout of exon 7 of the CaSR in various mouse models.[75,76] As yet, there are no animal models of FHH2 or FHH3.

2.5 Functional and Molecular Pathology

Because individuals with FHH1 have only one normal allele of the CaSR, there is a decrease in the activation of intracellular signaling at any given level of Ca_o^{2+}. In the parathyroid, this leads to a right shift in the setpoint for Ca_o^{2+}-regulated PTH secretion,[77] which "resets" the blood calcium concentration upward to varying degrees. This can be viewed as "resistance" of the parathyroid to Ca_o^{2+}, analogous to the forms of hormonal resistance seen with loss-of-function mutations of other receptors (e.g., thyroid hormone or androgen receptors).[78] In the kidney, in turn, there is blunted upregulation of renal Ca^{2+} excretion in response to hypercalcemia in FHH1 owing to renal resistance to Ca_o^{2+}.[79] Note that the CaSR indirectly regulates renal Ca^{2+} reabsorption by virtue of its control of PTH secretion, which, it turn, modulates renal Ca_o^{2+} handling. However, the abnormal regulation of renal Ca^{2+} excretion by Ca_o^{2+} persists in hypoparathyroid individuals with FHH, indicating that there is an intrinsic defect in renal Ca^{2+} handling in FHH.[79] Therefore, the abnormal Ca_o^{2+}-regulated renal Ca^{2+} excretion in FHH likely results not only from an intrinsic defect in renal Ca^{2+} handling but also from abnormal Ca_o^{2+}-regulated PTH secretion.

The elegant studies by Attie and coworkers more than 30 years ago homed in on the thick ascending limb of the loop of Henle as the "major renal locus of abnormal calcium transport" in this disorder, where hypocalciuria was PTH-independent and persistent despite coexistent hypercalcemia.[79] The reabsorption of Ca^{2+} in the TAL occurs as a result of a lumen-positive potential, driving divalent cation transport by the paracellular route. This positive transtubular potential difference is produced by the apical Na^+-K^+-$2Cl^-$ transporter (NKCC2), an apical K^+ channel, and a basolateral Cl^- channel (for review, see Ref. 6). The loop diuretic, ethacrynic acid, which inhibits the NKCC2, promoted renal excretion of calcium in hypoparathyroid subjects with FHH, an action that is known to take place in the cortical thick ascending limb.[79] The reduced function of the CaSR in the TAL in FHH, therefore, produces a failure to suppress the inappropriate paracellular reabsorption of Ca^{2+} (and Mg^{2+}) that persists in this condition in the face of hypercalcemia. This defect, however, can be bypassed by direct inhibition of NKCC2 by the loop diuretic. This clinical observation has been reproduced over 2 decades later by Egbuna, Brown and coworkers in observations made using the exon 5 CaSR knockout mouse models of disease.[80] All these observations point to the importance of

inactivation of the CaSR in the parathyroid and kidney and the contribution of TAL of the nephron in the anomalous renal handling of calcium in FHH1. Another consequence of the impaired Ca^{2+} sensing in the kidney is blunting of the usual hypercalcemia-induced reduction in the urinary concentrating mechanism. This observation is consistent with results of experiments in animals describing a role of the CaSR in acutely and chronically modulating vasopressin-regulated permeability and membrane transporters in the medullary thick ascending limb and inner-medullary collecting duct.[8]

To what extent can the phenotype of patients with FHH1 (namely, degree of hypercalcemia, elevation in PTH, etc.) be predicted from their genotype (e.g., the location of the corresponding mutation within the CaSR)? Although the extent of genotype–phenotype correlations in FHH1 uncovered to date is modest, some of the range of phenotypes seen in FHH can be explained, at least in part, based on available information about the biosynthesis, structure, and function of the cell surface form of the CaSR. The earliest stage at which mutations have been identified to interfere with the biosynthesis of the CaSR are two missense mutations in the signal peptide of the receptor, L11S and L13P (see http://www.casrdb.mcgill.ca for review of mutations), which fail to direct the nascent CaSR from the ribosome into the lumen of the ER.[81] These mutations, therefore, would not generate biologically active cell surface CaSRs even though the structure of the receptor after cleavage of the signal peptide would be totally normal where the protein fully translated. The remaining normal allele of the CaSR would presumably reach the cell surface and function normally in this setting, resulting in so-called haploinsufficiency, where a reduced quantity, presumably ~50%, of an otherwise normal protein arising from a single normal copy of the gene results in tissue dysfunction.

Another likely cause of haploinsufficiency with inactivating CaSR mutations is the introduction of a nonsense codon, producing a truncated receptor protein. Nonsense mutations are present throughout the CaSR protein, for example, beginning close to the receptor's amino-terminus (R25X, where X refers to a stop codon), as well as being present within the ECD (i.e., R185X or K323X), and TMD (R649X), but have not yet been reported in the C-tail (http://www.casrdb.mcgill.ca). An additional way in which the receptor protein can be truncated is through out-of-frame insertion and/or deletion mutations, which produce downstream stop codons [i.e., V268del/fsX273, which denotes a deletion (del) and frame shift (fs) producing a stop codon in the new reading frame at codon 273]. Mutations of this type are also present within the CaSR's ECD (V165del/fsX188) and TMD (P747ins/fsX776), as well as in the C-tail (X1079QextX1087) (http://www.casrdb.mcgill.ca). Like signal peptide mutations, truncation mutations, especially those within the ECD, which would lack the capacity to be anchored in the membrane and initiate intracellular signaling, would presumably simply result in a reduction in the quantity of normal receptor protein owing to the synthesis of the CaSR from only one wild-type CaSR allele. This would be comparable to the situation in the heterozygous CaSR knockout mice. In fact, heterozygous affected members of some FHH families with truncation mutations have been normocalcemic,[82] although this is not a universal finding.[57] In theory, receptor fragments long enough to include amino acids C129 and C131 could dimerize with the wild-type ECD and impact the function of the latter, but this has not be described and is of unknown functional consequences.

Most of the mutations causing FHH1 are missense mutations, and exhibit a range of inactivation, with EC_{50}'s for Ca^{2+} ranging from <twofold greater than that of the normal receptor to a total lack of activity (EC_{50} is the concentration of Ca^{2+} half maximally activating the receptor when expressed in heterologous cell systems, such as HEK293 cells). A substantial number of missense mutations within the CaSR's ECD are located close to the best characterized binding site for Ca^{2+} in the crevice between the two lobes of the VFT, comprising amino acids S147, S170, D190, Y218, and E297.[83] Only three of these mutations actually involve putative Ca^{2+}-binding residues (Y218S, Y218C, and E297K). It seems likely, however, that at least some of the other mutations, owing to their proximity to this or other Ca^{2+}-binding site(s) could reduce the affinity of the receptor for Ca^{2+} and/or interfere with the positive cooperativity needed for the steep relationship between Ca_o^{2+} and activation of the CaSR. Occasionally, one mutation at a specific residue can inactivate the receptor (P221S), while another activates it (P221L), suggesting that this residue is conformationally very sensitive with regard to the capacity of a specific mutated residue promoting the inactive versus active conformation.[84]

Many mutant CaSRs fail to reach the cell surface because their trafficking is blocked at various steps in the biosynthetic process. Breitwieser and coworkers [85] have categorized these into type Ib, which cannot exit the ER, and type Ia, which traffic to the Golgi but no further. Type II mutants reach the cell surface and were categorized into type IIa, whose activity could be enhanced by the calcimimetic NPS R-568, and type IIb, which were inactive with or without NPS R-568. Type I mutants are effectively null mutants and could conceivably interfere with the biosynthesis of the wild-type receptor, for example, by heterodimerizing with it in the ER and potentially preventing it from trafficking forward in the biosynthetic pathway, although this hasn't been reported.

Because the cell surface CaSR functions as a dimer, one must consider possible dominant negative effects of type II mutants when assessing the potential impact

of mutant receptors on the wild-type CaSR. For mutant CaSRs reaching the cell surface, statistical considerations would suggest that 25% of the receptors in parathyroid and kidney cells are wild-type homodimers, 25% are mutant homodimers, and 50% are mutant-wild-type heterodimers. Assuming that mutant homodimers function poorly or at all as physiological levels of Ca_o^{2+}, for this repertoire of receptors to signal equivalently to the 50% complement of normal CaSRs thought to be present with haploinsufficiency, the 50% mutant-wild-type heterodimers would need to signal with the same efficiency as the 25% wild-type homodimers. Otherwise, if the mutant partner in the heterodimers did not negatively impact its wild-type partner, the percentage of normally functioning receptors in this scenario would be 75% (i.e., everything except the mutant homodimers). Such a complement of normally functioning receptors might perform even better than in the setting of null mutations (~50% of the normal quantity of wild-type CaSR), which is not generally seen. This may imply, therefore, that most type II mutant CaSRs present in wild-type-mutant heterodimers interfere to some extent with the function of their normal partner (i.e., exerts a dominant negative effect). In fact, more pronounced dominant negative effects of some mutant CaSR's (e.g., S137P, R185Q, R227L, R795W, and F881L) (http://www.casrdb.mcgill.ca) have been described in vitro, which can be associated with more pronounced hypercalcemia in vivo than is the norm in FHH1.[58] Fig. 37.3 shows the dominant negative impact of this mutation when studied in vitro.[58] Infants with this mutation are likely to present as NSHPT/NHPT and affected individuals of all ages can manifest serum calcium concentrations of 13–14 mg/dL.[29,58] A more recently described genotype–phenotype relationship of unknown mechanism is the presence of hypercalciuria in persons harboring missense mutations in C-tail of the CaSR.[56,86] It has been suggested that mutant receptors of this type couple more effectively to downstream effects in kidney than in parathyroid, producing greater "resistance" to Ca_o^{2+} in the latter than the former.[3]

Regarding parathyroid histopathology in FHH, the largest available studies, carried out at a time when parathyroidectomy was more common because FHH had not yet been clearly distinguished from PHPT, reported either normal sized glands, with a tendency to an increased percentage of fat cells relative to normal glands,[87] or mild parathyroid hyperplasia, with variably enlarged parathyroid glands, sometimes to a substantial degree.[88] More recent studies have described the presence of single and multiple parathyroid "adenomas" in several patients with inactivating CaSR mutations, as noted earlier,[55,56] or, in some cases, chief cell or lipohyperplasia (hyperplasia of both chief and fat cells) with one or more enlarged glands resembling adenomas.[89] Note that these studies were carried out prior to an understanding of the molecular causes of FHH and so histology was not linked to the various forms of FHH, although most of these patients likely had FHH1. To our knowledge, parathyroid histology is not available for FHH2 or FHH3.

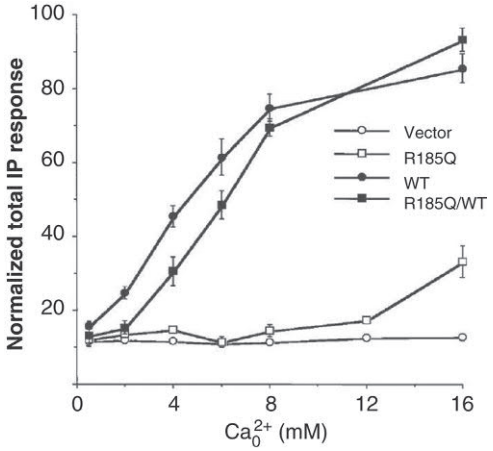

FIGURE 37.3 Demonstration of the dominant negative effect of mutation R185Q in the CaSR ECD. Note that when the wild type (WT) and mutant receptors are coexpressed, the presence of the mutant receptor shift the concentration-response curve of the wild- type receptor to the right (e.g., it takes a higher concentration of Ca_o^{2+} to activate the receptor to the same extent). CaSR-mediated stimulation of phospholipase C, as manifested by the resultant increase in total inositol phosphates (IP). *Source: Reproduced from Bai M, Pearce SH, Kifor O, et al. In vivo and in vitro characterization of neonatal hyperparathyroidism resulting from a de novo, heterozygous mutation in the Ca2+-sensing receptor gene: normal maternal calcium homeostasis as a cause of secondary hyperparathyroidism in familial benign hypocalciuric hypercalcemia.* J Clin Invest *1997;***99***(1):88–96, with permission.*

Based on molecular modeling, the mutations in *GNA11* causing FHH are all predicted to disrupt GDP turnover, and therefore, interfere with the ability to exchange/release GDP from the inactive GDP-bound form of the G protein, which is normally followed by binding of GTP and activation of the G protein.[36,70] As might be expected, when coexpressed with the normal CaSR in HEK293 cells, the mutant G proteins cause a modest rightward shift in the activation of Ca_i^{2+} signaling by increases in Ca_o^{2+}, similar to the "resistance" to Ca_o^{2+} observed when mutant CaSRs from patients with FHH1 are expressed in the same in HEK293 cells with a normal complement of endogenous G proteins. The similar degree of Ca_o^{2+} resistance in vivo in patients with FHH1 and FHH2 owing to the presence of mutant CaSRs and $G\alpha_{11}$, respectively, would lead to the deranged control of PTH secretion and renal tubular calcium reabsorption observed in these two forms of FHH. The modest impact of the transfected mutant G protein on Ca_o^{2+}-evoked Ca_i^{2+} signaling likely contributes importantly to the mild phenotype in FHH2. It may not be simple, however, to extrapolate these results to what is observed in vivo in parathyroid and kidney, where there are two normal $G\alpha_q$

alleles and one normal $G\alpha_{11}$ allele to compensate for the one mutant $G\alpha_{11}$ allele.

The precise role of AP2S1 in the function of parathyroid gland and kidney (e.g, in the control of renal Ca^{2+} reabsorption) is not clear. However, the mutant σ-1 exerts clear actions when cotransfected with wild-type CaSR into HEK293 cells, despite the presence of normal AP2S1 in the cells. There is a right shift in the relationship between Ca_o^{2+} and associated increases in Ca_i^{2+}, as well as a reduction in high Ca_o^{2+}-evoked internalization of the wild-type CaSR.[38] The relationship between Ca_o^{2+} and Ca_i^{2+} exhibits a clear dominant negative action of the transfected AP2S1,[67] which is very likely to be an important contributor to the more severe hypercalcemia observed in FHH3 families compared to most FHH1 kindreds, as well as the limited number of FHH2 kindreds studied to date. The significance of the reduced internalization of the wild-type CaSR when cotransfected with cDNA encoding mutant AP2S1 is uncertain. One might have expected that an increase in cell surface CaSR might enhance rather than diminish Ca_o^{2+}-evoked Ca_i^{2+} signaling. This result indicates that there isn't a simple 1:1 relationship between the level of cell surface expression of the CaSR and its capacity to activate cellular signaling, at least signaling via G_q/G_{11} and Ca_i^{2+}. Internalized GPCRs con contribute to agonist-induced signaling in some cases, and perhaps a similar mechanism is at play here.

2.6 Diagnostic Aspects

The characteristic, overly avid renal tubular reabsorption of calcium in FHH is most readily apparent when expressed as the calcium to creatinine clearance ratio, calculated as shown in Eq. 37.1:

$$(\text{Urinary calcium}/\text{plasma calcium}) \times (\text{plasma creatinine}/\text{urinary creatinine}) \quad (37.1)$$

In practice, about 80% of patients with FHH1 have clearance ratios of <0.01 [42] or <0.115 in a later study,[90] and a similar percentage of patients with PHPT have values >0.01. Thus a clearance ratio of less than 0.01 does not exclude the 10%–20% of cases of PHPT with a clearance ratio <0.01, and a value greater than this level does not exclude FHH. It has recently been demonstrated that clearance ratios less than those in FHH1 and FHH2 are commonly observed in FHH3.[40,67] Factors that can contribute to hypocalciuria in patients other than those with FHH with PTH-dependent hypercalcemia, such as PHPT, and should be ruled out include very low calcium intake, vitamin D deficiency, the use of thiazide diuretics or lithium and mild renal insufficiency ($\sim \geq$ 50% reduction in creatinine clearance). The importance of differentiating between FHH and PHPT lies in the propensity of patients with the latter to develop complications, including bone loss, impaired renal function, kidney stones, and others, which increase in frequency with the duration of the disease,[91] in contrast to the generally benign clinical course in FHH. Moreover, parathyroid surgery is generally curative in PHPT, while most, but not all,[56] patients with FHH undergo recurrence of their hypercalcemia,[42,54] an undesirable outcome, particularly in a benign condition.

The recent discoveries of the molecular bases for FHH2 and FHH3 require a reassessment of the appropriate testing that should be carried out to establish the cause of FHH in a given individual and family. Genetic testing for inactivating mutations in the *CASR*, *GNA11*, and *AP2S1* genes provides a definitive diagnosis of FHH1, FHH2, and FHH3, respectively. If feasible, it is useful if a mutation is also shown to be inactivating by expression studies to prove that it is not simply a benign polymorphism. A useful algorithm has recently been proposed for establishing the molecular diagnosis in patients suspected of having FHH as a group or PHPT on the basis of hypercalcemia and a normal/raised PTH (Fig. 37.4).[67] An initial evaluation of the patient involves calculation of a calcium to creatinine clearance ratio. Those with a clearance ratio >0.02 are less likely to have FHH and more likely to have PHPT. If the clearance ratio is in the indeterminate range of 0.01–0.02, genetic testing is needed to make a definitive diagnosis of FHH1, FHH2, or FHH3. The distinction between FHH3 and FHH1 and FHH2 may be feasible using a parameter developed by Hannan et al.[67] called the CMCR index, which is (serum calcium × serum magnesium)/(100 × calcium to creatinine clearance ratio). A value of >5 is diagnostic of FHH3 with a sensitivity of 83% and specificity of 86%. Genetic testing in such cases can be limited to the AP2S1 gene. If CMCR is < 5, testing should be carried out for all three FHH genes.

A syndrome that can closely mimic FHH is autoimmune hypocalciuric hypercalcemia (AHH), which results from inactivating antibodies directed at the CaSR that reduce the level of activity of the receptor at any given level of Ca_o^{2+}. A handful of such cases have been described.[34,92–94] In general, these individuals have one or more additional autoimmune conditions affecting other organs, such as antithyroid antibodies in Hashimoto's thyroiditis. In a patient who has what appears to be FHH but has one or more autoimmune manifestations, this condition should be suspected. Testing for the presence of such anti-CaSR antibodies is not widely available but may be obtained on a research basis in laboratories studying AHH. Presumably the anti-CaSR antibody/ies stabilize an inactive conformation of the CaSR. As in FHH, surgery may not cure the hypercalcemia in patients with AHH,[92] and based on our current understanding of this syndrome should be avoided. In one case, the hypercalcemia in AHH was steroid-responsive,[92] but in another it was not.[93]

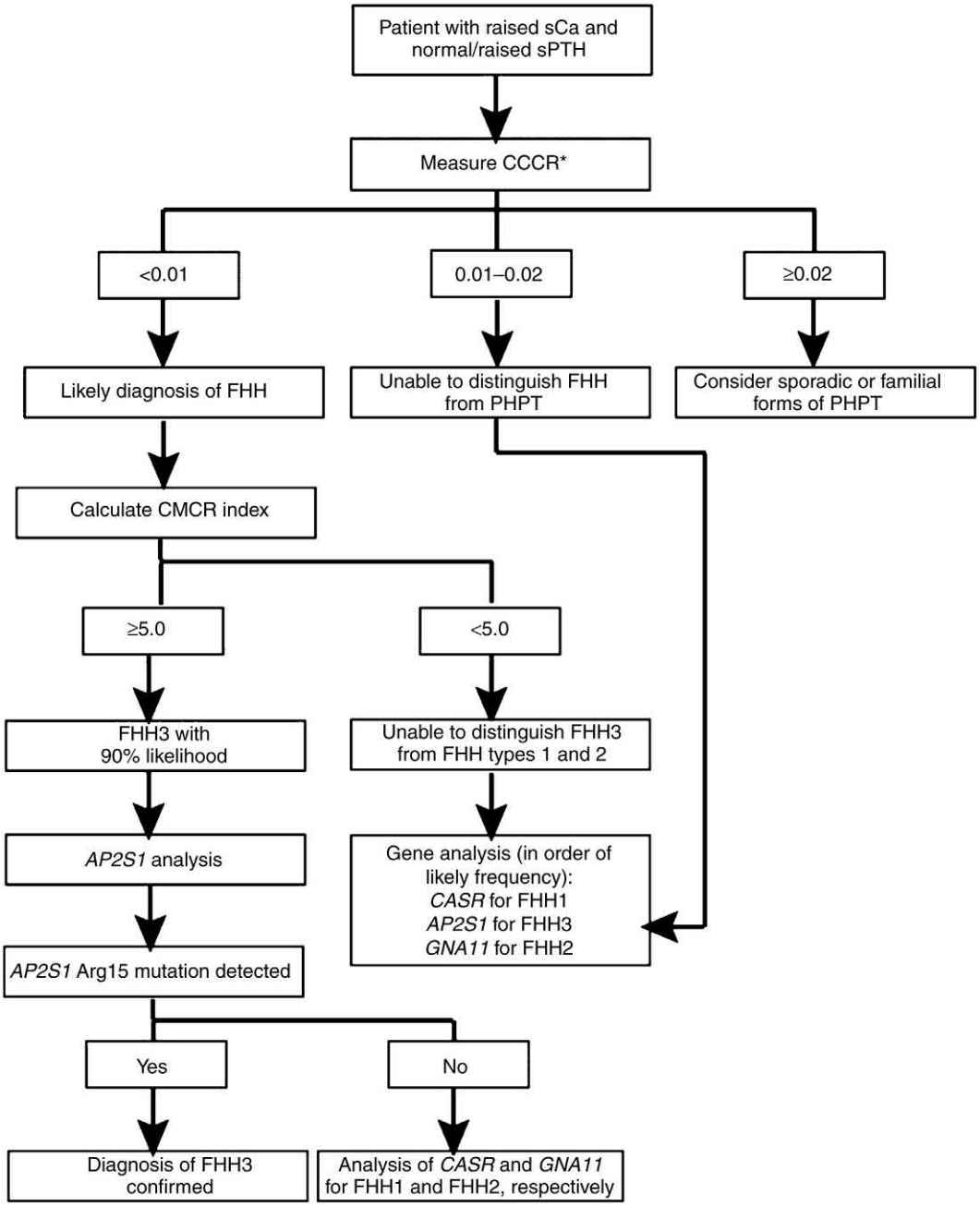

FIGURE 37.4 Suggested algorithm for evaluating patients with PTH-dependent hypercalcemia suspected of having FHH and for differentiating among FHH1, FHH2, and FHH3 (see text for details). Source: Reproduced from Hannan FM, Howles SA, Rogers A, et al. Adaptor protein-2 sigmasubunit mutations causing familial hypocalciuric hypercalcaemiatype 3 (FHH3) demonstrate genotype-phenotype correlations, codon bias and dominant-negative effects. Hum Mol Genet2015;24(18):5079–92, with permission.

2.7 Counseling

It is important that individuals with the three forms of FHH are aware of and understand the nature of their conditions. In families with classic biochemical findings (e.g., a calcium to creatinine clearance ratio <0.01, normal PTH, and mild to moderate hypercalcemia), the risk of long-term complications is low. Affected family members other than the proband should be identified and the patients' and other caregivers need to be made aware that the diagnosis is FHH and not PHPT. Parathyroid surgery is contraindicated in most such cases. Some CaSR mutations, however, can lead to more severe hypercalcemia, hypercalciuria/nephrolithiasis, parathyroid adenoma(s) and/or hyperplasia, and pancreatitis (vide supra). Moreover, it has recently been recognized that AP2S1 mutations are associated with a more severe phenotype.[40,67] Therefore, a blanket recommendation

that parathyroid surgery should be avoided is inappropriate, and cases with more severe phenotypes should be evaluated on a case-by-case basis. This is particularly true in patients with FHH1 who have overt hyperparathyroidism, in whom removal of enlarged/adenomatous parathyroid glands can substantially reduce or normalize serum calcium concentration.[55,56] Similar considerations regarding FHH3 await further information about the clinical characteristics and natural history of these less common forms of FHH, especially the greater severe of hypercalcemia in some of these patients and other worrisome phenotypic features, such as reduced BMD.[40,67]

2.8 Treatment

As just noted, the classic recommendation of avoiding parathyroid surgery in FHH is not universally true. As more is learned about the range of phenotypes and natural histories of FHH1, FHH2, and FHH3 the need for surgery in specific settings should become clearer, although the great majority of patients with FHH1 and presumably, FHH2 clearly do well with just watchful waiting. An additional form of either short- or long-term treatment of cases who might benefit from parathyroid surgery but in whom surgery is contraindicated or refused would be the use of a calcimimetic, as recently described in a patient with NHPT.[95] Many mutations in the CaSR in FHH1 retain some degree of responsiveness to calcimimetics, and even in the case of nonsense mutations unable to respond to the drug, the remaining normal allele will show a left shift in its concentration response curve during treatment.[96] Of considerable interest, cinacalcet has recently been shown to improve the functional abnormalities in FHH2 [97] in vitro and FHH3 in vivo,[98] and provides, therefore, a potentially useful medical therapy in selected patients.

3 CLINICAL AND GENETIC FEATURES OF NEONATAL SEVERE PRIMARY HYPERPARATHYROIDISM [OMIM 239200]

3.1 Clinical Description

In most cases, there is a clear gene dosage effect for inactivating mutations of the CaSR: heterozygous inactivating mutations leading to FHH with mild-to-moderate hypercalcemia (albeit occasionally producing NHPT or even NSHPT) and homozygous [99,100] or compound heterozygous mutations [82] resulting in NSHPT. The latter is a much more severe, potentially fatal, condition and presents most commonly within the first 6 months of life. Infants with NSHPT can exhibit one or not uncommonly, multiple fractures, polyuria, dehydration, hypotonia, and failure to thrive.[99,101,102] Biochemical evaluation typically shows severe hypercalcemia and hyperparathyroidism, which can be accompanied by relative hypocalciuria.[102] In homozygous cases of NSHPT, total serum calcium concentrations are usually greater than 15 mg/dL and can exceed 30 mg/dL in the most severely affected cases.[101] PTH levels are not infrequently 5–10-fold the upper limit of normal. The mass of the parathyroid glands in NSHPT is generally increased several-fold, and they exhibit prominent chief cell hyperplasia.[101,102] A striking feature of the disease is the associated hyperparathyroid bone disease, which can manifest with the radiological findings of rickets, skeletal undermineralization, and/or subperiosteal resorption.[102,103] In addition to fractures of long bones, infants with NSHPT can have multiple rib fractures, which can, in some cases, produce a "flail chest" syndrome that causes respiratory difficulties, owing to a decreased capacity of the affected infant to expand its chest wall and generate the negative intrathoracic pressure needed for normal respiration.[103] Early diagnosis is critical in severe cases, as untreated NSHPT, in addition to causing morbidity related to the severe hypercalcemia, hyperparathyroidism and bone disease, can be a devastating neurodevelopmental disorder,[104] and in some cases can be lethal without parathyroidectomy to alleviate the hyperparathyroidism and hypercalcemia.[101,102] However, with improved supportive therapy prior to parathyroid exploration, mortality in severe cases has substantially declined over the past 2–3 decades. Moreover, as will be described later, the ability to document the presence of *CASR* mutations by direct sequencing has considerably broadened the range of severity of diseases associated with homozygous (or compound heterozygous) inactivating CaSR mutations. To date, no cases of NSHPT caused by homozygous mutations in *GNA11* or *AP2S1* have been described, perhaps because of embryonic lethality due to the key roles of these genes in multiple tissues. Therefore, the discussion that follows will be limited to cases of NSHPT and NHPT in the setting of FHH1.

3.2 Genetics

In 1982, more than 10 years prior to the identification of heterozygous inactivating mutations of the CaSR as the cause of most cases of FHH, Marx and coworkers described three families with FHH in whom there were one or more cases of NSHPT.[99] They suggested that the two syndromes could be genetically related, with NSHPT in some cases representing the homozygous form of FHH. Shortly after the cloning of the CaSR and the identification of heterozygous inactivating mutations in FHH, Pollak et al. studied 11 kindreds with FHH, in whom consanguineous unions engendered 4 infants with NSHPT who were homozygous for inactivating CaSR mutations, documenting that homozygosity for such mutations is

a key cause of NSHPT.[100] It should be recognized, however, that, owing to the infrequency of consanguinity, NSHPT as a result of homozygous CaSR mutations is quite uncommon in FHH families considered as a whole.

3.3 Molecular Genetics

At least 42 mutations of the CaSR have been described in NHPT or NSHPT (http://www.casrdb.mcgill.ca/cgi-bin/casrdb/casrdb_mutQ1.cgi). In 5 cases the same mutation is present in more than one, presumably unrelated, family. Twenty six (61%) are in the ECD [similar to the percentage of mutations in the ECD in FHH (~75%)], 4 are in the intracellular loops and 4 in the extracellular loops. None of the reported mutations causing NSHPT are present in the TMD or in the C-tail, although a family has been described with family members having NSHPT due to a homozygous Alu element inserted into the proximal C-tail.[105] Twenty four of the mutations causing NSHPT are missense (58%), 8 are nonsense (19%), 3 are insertions/deletions producing frame shifts and premature stop codons (14%), and 1 is a deletion/insertion without a frame shift. Relative to the mutations described in FHH, a lower percentage are missense mutations, and greater percentages are nonsense mutations and insertion/deletion mutations, but the number of mutations described to date in NSHPT is relatively small, and it remains to be seen whether these patterns will continue as more mutations are described. Note that the mouse models of NSHPT were described earlier in the Section 2.4.

3.4 Functional and Molecular Pathology

Homozygous nonsense mutations and insertion/deletion mutations producing premature stop codons would be expected to abolish or severely impair CaSR function. Most of the remaining missense mutations causing NSHPT presumably result in a sufficient increase in EC_{50} to produce severe hypercalcemia in the homozygous state. For example, a twofold increase in EC_{50} might be expected to cause as much as a twofold increase in serum calcium concentration (e.g., ~20 mg/dL) when present in the homozygous state, since the CaSR's EC_{50} is an important determinant of the level at which the extracellular calcium concentration is "set" in vivo. In contrast, as noted below, cases have been described in which homozygous inactivating mutations produced relatively small increases in serum calcium concentration ($\leq$15 mg/dL) that were compatible with life, in association with EC_{50}'s that were significantly less than twofold above that of the wild-type receptor.[44]

The genotype–phenotype relationships for homozygous inactivating CaSR mutations have been clarified considerably over the past 10–15 years, including the relationships between heterozygous and homozygous inactivating CaSR mutations and the severity of disease. As described earlier, not all cases diagnosed with NSHPT harbor homozygous mutations in the receptor. Infants harboring mutations exerting a prominent dominant negative (e.g., R185Q) [58] can present as NSHPT, with severe hyperparathyroidism and bone disease, albeit with serum calcium concentrations lower than in most patients with homozygous FHH (e.g., <15 mg/dL). Several cases of NHPT/NSHPT have had de novo, heterozygous missense mutations located in the ECD, with no mutation found in the parents.[57,58] One individual with de novo NHPT/NSHPT was heterozygous for a previously described mutation in an FHH family (R185Q) [58] that was associated with unusually high serum calcium concentrations for FHH owing to the dominant negative action of the mutant CaSR on the wild-type partner in mutant-wild-type heterodimers.

More recently recognized clinical presentations of homozygous CaSR mutations in addition to NSHPT that are associated with less inactivation of the CaSR include: (1) a child with moderate hypercalcemia (12–13 mg/dL) who was homozygous for the CaSR mutation, Q459R, and effectively presented in an autosomal recessive manner, since both related parents and five of six other heterozygous family members were normocalcemic;[44] (2) homozygous individuals who were only serendipitously diagnosed as adults. Two such adults were 35- and 26-years old at the time of diagnosis, and appeared asymptomatic with normal renal function despite serum calcium concentrations of 15–17 mg/dL.[106,107] The two mutations in these cases were P39A and Q27R, respectively, and they exhibited only modest increases in EC_{50} of 4.4 and 4.9 mM (vs. 3.7 mM for the normal receptor). In view of their asymptomatic state, parathyroid surgery was not entertained in these two cases; and (3) a homozygous adult who presented with bilateral renal calculi, osteoporosis, and a biochemical picture of PHPT [serum Ca^{2+} of 15 mg/dL, PTH of 65 pg/mL (normal range in this laboratory <30 pg/mL), and calcium to creatinine clearance ratio of 0.024].[55] After removal of 2 adenomas, her serum calcium remained elevated at 12 mg/dL, presumably because of the presence of the mildly inactivated, homozygous mutant CaSR in the remaining parathyroid glands and kidney. Finally, rare cases of NSHPT have resulted from the presence of compound heterozygous inactivating mutations of the CaSR. In one such case, there was a mutation in exon 7 from the mother and a mutation in exon 4 from the father thereby resulting in a total absence of normal CaSRs and the clinical presentation of NSHPT.[82] Another case of NSHPT due to compound heterozygosity for two different truncation mutations has been reported more recently.[108]

Prominent pathological features of NSHPT are the associated hyperparathyroid bone disease, with radiological and histological findings that can include demineralization and subperiosteal resorption, as well as rachitic changes, accompanied by multiple factures of long bones and ribs.[99,101–103] Regarding the histopathology of the markedly enlarged parathyroid glands in NSHPT, there is most commonly prominent chief cell hyperplasia in NHPT and NSHPT.[3,102] In occasional cases, there is a microscopic picture of lipohyperplasia,[89] in which there is hyperplasia of both chief cells and fat cells. Whether specific mutations are associated with pure chief cells hyperplasia, on the one hand, or lipohyperplasia, on the other, is not currently known.

3.5 Diagnostic Aspects

Recognition of the characteristic clinical, radiological, and biochemical features of NSHPT does not usually produce a diagnostic dilemma, although, as discussed earlier, some cases of homozygous FHH1 have escaped clinical detection, and such cases may only be identified later in childhood or in adulthood. Fortunately, in most cases the clinical sequelae of a delayed diagnosis in these milder cases do not appear to be severe. It should be emphasized, however, that in clinically severe NSHPT, the consequences of delayed diagnosis could be devastating.[104]

Inactivating mutations of the CaSR are arguably the most common cause of PTH-dependent hypercalcemia in neonates and children under age 10. Other hereditary causes of hyperparathyroidism that can be encountered in childhood, such as multiple endocrine neoplasia type 1 and the hyperparathyroidism-jaw syndrome, usually present in children over the age of 10.[54] Therefore, mutational analysis of the CaSR should be carried out in any child under the age of 10 with PTH-dependent hypercalcemia. Clearly, if a mutation is found, other family members should be screened in order to determine whether the mutation is inherited or de novo and to identify those individuals who should undergo genetic counseling. Mutational analysis of cases with less severe manifestations of homozygous CaSR mutations will identify cases, which escaped diagnosis in childhood. The recognition that homozygous CaSR mutations can rarely present with findings typical of PHPT (e.g., PTH-dependent hyper- rather than hypocalciuria) dictates that the clinician should be alert to this possibility.[55]

3.6 Counseling

The discovery of homozygous or compound heterozygous mutations in the CaSR in a child should prompt biochemical (e.g., measurement of serum calcium concentration) and mutational screening of the parents. If hypercalcemia is a reliable indicator of the presence of the mutation, it can then be used to screen other family members, who should be made aware of their diagnosis for the reasons described earlier for FHH. If the parents desire additional children, they should be informed of the 25% chance of having another homozygous or compound heterozygous child. Prenatal diagnosis should be at least theoretically feasible in such cases, and has been used in a pregnancy of an individual with an activating mutation of the CaSR,[109] although not for prenatal diagnosis of FHH to our knowledge. Individuals homozygous or compound heterozygous for inactivating CaSR mutations, who survive to adulthood, with or without parathyroidectomy, and are capable of bearing children should be made aware that all of their children will have FHH or one of its variants.

3.7 Treatment

Surgery remains an important therapeutic modality in cases of NSHPT with marked hypercalcemia (e.g., >15–20 mg/dL). If possible, it is desirable to stabilize the patient first using vigorous hydration and judicious use diuretics given that these patients may be dehydrated at presentation.[101] The use of a bisphosphonate, to stabilize such patients prior to parathyroid surgery, is a more recent addition to the therapeutic armamentarium and may produce a significant, beneficial drop in serum calcium concentration, although the effect is usually temporary.[92,93] The use of the calcimimetic, cinacalcet, has recently been reported to decrease serum calcium concentration in several cases of NHPT or NSHPT,[95,110] although it is not approved for this purpose. In homozygous cases, the drug would only be expected to work if the mutant receptor retained some degree of function that was improved by the calcimimetic. In one report, for example, treatment of a patient with NSHPT owing a truncation mutation of the region of the CaSR thought to bind the drug, showed no response to therapy with cinacalcet.[111] In heterozygous cases of NHPT, the drug could provide benefit by positively impacting the function of the mutant CaSR and, perhaps, by left-shifting the concentration-response curve of the remaining normal CaSR.[95]

If hypercalcemia remains life-threatening, surgery should usually be undertaken, usually total parathyroidectomy, if possible, as any remaining parathyroid tissue will retain the abnormal functional properties imparted by the mutant CaSR(s). Cryopreservation of resected parathyroid tissue can be performed, although its functional properties will also be abnormal. Milder cases of NSHPT, particularly those caused by heterozygous mutations, may revert to a clinical and biochemical picture of FHH, with healing of bony lesions and stabilization of serum calcium concentration at a level comparable to the other affected members in that kindred with, and also, in some cases, without parathyroid surgery.

4 CLINICAL AND GENETIC FEATURES OF AUTOSOMAL DOMINANT HYPOPARATHYROIDISM CAUSED BY ACTIVATING MUTATIONS OF THE CaSR [OMIM - #6011298, ADH TYPE 1 (ADH1)] OR OF *GNA11* [OMIM - #615361, ADH TYPE 2 (ADH2)]

4.1 Clinical Description

Autosomal dominant hypoparathyroidism [(ADH; sometimes referred to as autosomal dominant hypocalcemia or autosomal dominant hypercalciuric hypocalcemia (ADHH)] due to activating mutations of the CaSR or $G\alpha_{11}$ is one of several forms of hereditary hypoparathyroidism. These can be caused by mutations in genes impacting parathyroid gland development or function, including the gene encoding the parathyroid-specific transcription factor *Glial cells missing B* (GCMB),[112] the *PTH* gene,[113] the *CaSR* gene (now known as ADH1), or the *GNA11* gene (ADH2). An updated list of activating mutations of the CaSR that cause ADH1 is available at http://www.casrdb.mcgill.ca). The most recently described of these disorders, ADH2, results from germline gain-of-function mutations in the *GNA11* gene encoding for $G\alpha_{11}$ with linkage to 19p13.3,[36,37] ADH can also be a part of the HDR (hypoparathyroidism, deafness, and renal anomaly) syndrome due to a dominant negative mutation of the *GATA3* gene.[114] In contrast to the other forms of hypoparathyroidism just noted, where parathyroid function is severely impaired or absent, ADH1 and ADH2 are regulatory defects, in which the setpoint for the control of parathyroid function and renal calcium handling by Ca_o^{2+} is reset downward to maintain a relative constant, but subnormal, level of Ca_o^{2+}. To date, no cases have been described as a result of activating mutations of AP2S1.[115]

ADH1 due to activating mutations of the CaSR, although rare, in index cases may comprise a sizeable fraction of cases of idiopathic hypoparathyroidism, perhaps representing as many as a third of such cases.[116] Patients with ADH1 are commonly asymptomatic, and the condition clinically and biochemically mimics autoimmune hypoparathyroidism. Because of the range of phenotypic manifestations of ADH1 due to CaSR mutations, the exact prevalence, incidence, or geographic distribution of this condition is unknown.

Patients with ADH1 generally exhibit mild to moderate and, occasionally, severe hypocalcemia (as low as 4.5–5 mg/dL), with serum PTH levels that are inappropriately low given the level of hypocalcemia, for example, within the lower half of the normal range or frankly subnormal.[116–118] Serum phosphorus levels, as in other forms of hypoparathyroidism, are elevated, roughly in an inverse proportion to the degree of hypocalcemia. Serum magnesium levels are not uncommonly low, particularly in ADH1.[118] Affected individuals often exhibit relative or absolute hypercalciuria, with normal or frankly elevated urinary calcium excretion, respectively, in spite of their low-serum calcium concentration, which can be accompanied by nephrocalcinosis, nephrolithiasis, and renal impairment, particularly in the setting of treatment with calcium and vitamin D supplementation.[116,118] Some patients, especially children during febrile episodes, can exhibit neuromuscular irritability with carpopedal spasm, muscle cramps, and/or seizures, and in some cases calcification of the basal ganglia may be present.[116,118] In a recent report of a long-term survey of 25 German patients with ADH1, a range of phenotypic manifestations were found: 50% of patients were symptomatic, 36% had basal ganglia calcification and 12% had nephrocalcinosis.[117] The six individual cases or families with ADH2 studied to date, have mild to moderate hypocalcemia, but commonly have symptomatic neuromuscular irritability, including seizures, and as in ADH1, can develop complications of therapy with calcium and vitamin D, such as nephrolithiasis.[119] Untreated ADH2 patients, however, may have less hypercalciuria than those with ADH1. Some ADH2 kindreds have been reported to have short stature, presumably due to noncalciotropic actions of the activated G protein.[120]

4.2 Genetics and Molecular Genetics

Soon after the cloning of the CaSR, investigators[121] showed linkage of ADH to a locus on chromosome 3 q13—the same locus containing the gene for the CaSR. Nearly simultaneously, a heterozygous missense mutation, Q127A, was shown to be the cause of ADH in an unrelated family.[33] Since these first reports, at least 95 CaSR mutations have been characterized that cause ADH1 (http://www.casrdb.mcgill.ca). Of these, 92 are missense mutations, 2 are deletions and 1 is an insertion mutation. Of these mutations leading to ADH1 a majority occur in the ECD. Ninety-two are missense mutations, two are deletions, and one is an insertion mutation. (CASRDB- http://www.casrdb.mcgill.ca/?Topic=CasrMutation&v=new). Note that an activating mutation can be present at an amino acid residue that, when mutated to a different amino acid, can be inactivating, suggesting a location in the receptor sensitive to small changes in conformation associated with the presence of a different amino acids at the site. In addition, rare cases have in-frame deletions or insertion/deletion mutations with a frame shift and resultant premature stop codon. Most ADH1 patients are heterozygous for the activating mutation. In one family, a homozygous mutation was described, but it was not associated with a more severe phenotype.[122] Although there is a spectrum of phenotypic severity for a given genotype, the symptoms present in affected members of the same family tend to be similar.

To date only six $G\alpha_{11}$ mutations causing ADH2, all missense, have been reported.[36,37,119,120] The first two reports of ADH2 as a result of *GNA11* mutations were published simultaneously in the same journal, one describing two probands and the second two kindreds with a total of four distinct mutations (R181Q and F341L [36] and R60C and S211W [37]) A third report in 2014 described another family with an activating mutation of $G\alpha_{11}$ (R60L).[120] More recently, Piret, Gorvin and coworkers investigated a multigenerational nonconsanguineous family, from Iran, with ADH and keratoconus which are not known to be associated, for causative mutations by whole-exome sequencing in two individuals with hypoparathyroidism, of whom one also had keratoconus, followed by cosegregation analysis of variants.[119] This identified a novel heterozygous germline V340M $G\alpha_{11}$ mutation in both individuals, which was also present in the other two relatives with hypocalcemia that were tested.

4.3 Animal Model

An activating mutation is present in the CaSR of the *Nuf* mouse, which was created as part of a mutagenesis experiment.[123] The mice are superficially normal appearing but have widespread calcification, especially of striated, cardiac and smooth muscle, as well as blood vessels, and develop cataracts spontaneously. The mutation produces a biochemical phenotype of hypocalcemia, hyperphosphatemia, and low PTH. Homozygous Nuf/Nuf mice are more severely affected than heterozygotes (Nuf/−), with about a 30% lower serum calcium concentration in the former. An unexpected phenotype of the Nuf mice is sudden death without any apparent pathology that might cause death. This phenotype occurred mainly in lactating females, suggesting that the added loss of calcium in the milk may have precipitated more severe hypocalcemia and death due to arrhythmias or other causes. The mutant CaSR, L723Q (numbered according to the sequence of the murine CaSR), replaces a highly conserved hydrophobic residue in the TMD with a more hydrophilic residue. When this mutation was engineered into the human CaSR (wild-type mouse and human CaSR both have L723), the mutated receptor showed a left shift in its response to increasing concentrations of Ca^{2+}.[123] Thus while there are differences, as well as similarities between the Nuf mouse and patients with ADH1, it provides an interesting and potentially useful model of this condition. Of note, the parathyroids of the Nuf mice are normal in gross appearance and histologically.[123]

4.4 Functional and Molecular Pathology

Patients with ADH1 harbor an activating mutation in the *CASR* gene that resets the setpoint of Ca_o^{2+}-regulated PTH secretion leftward and lowers renal calcium reabsorption at any given level of serum calcium. When expressed in heterologous systems, these mutations universally cause a left shift in the activation of the CaSR by Ca_o^{2+};[33,118,124] they only rarely induce constitutive activation of the receptor.[125] The mechanisms by which ADH1 mutations cause increased sensitivity of the CaSR to Ca^{2+} are being actively investigated. Many questions remain but a few tentative conclusions can be drawn. At least 16 of the mutations in the ECD are clustered between residues 116 and 131 of the CaSR, and at least 8 involve cysteines 129 or 131, which are known to be involved in dimerization of the CaSR,[25] suggesting that this region and these two disulfide bonds contribute to maintaining the receptor in an inactive state (http://www.casrdb.mcgill.ca). The other mutations in the ECD could potentially result in conformational changes that increase the affinity of the CaSR's ECD for Ca^{2+} or favor the active conformation of the ECD. It is thought that changes in the orientation of the transmembrane helices, as well as in the intracellular loops and C-tail contribute to transmission of an extracellular signal into activation of its G proteins by GPCRs. Mutations in the transmembrane helices and intracellular loops of the CaSR may produce conformational changes that activate the receptor in this way. One case of ADH1 was caused by mutation of a protein kinase C (PKC) phosphorylation site in the CaSR's C-tail to a methionine (T888M).[126] Since phosphorylation of this site exerts negative feedback regulation of the CaSR, loss of the tonic inhibitory action of PKC on the receptor presumably causes ADH1.

Two of the deletion/insertion mutations in the C-tail of the CaSR, one with or one without the generation of a premature stop codon, as well as the missense mutation R898Q, are of particular interest. All three disrupt an arginine rich region (R896/R897/R898) noted earlier that serves as an ER retention signal,[26] and may, therefore, promote increased cell surface expression of the receptor. Indeed, expression of the mutant receptor with a deletion between residues 895 and 1075, markedly increases cell surface expression relative to the wild-type receptor [122] (Fig. 37.5). The increase in cell surface expression could increase sensitivity to Ca_o^{2+}, because there would be a greater total number of activated receptors at any given level of Ca_o^{2+} and, consequently, more efficient stimulation of signal transduction. This family with the in-frame deletion within the C-terminus of the CaR contained the only individual to date known to be homozygous for an activating mutation, whose phenotype, as noted earlier, was very similar to that of the heterozygous family members. Thus one mutated allele may be enough to induce a maximal shift in the setpoints of Ca_o^{2+}-regulated PTH secretion and renal Ca^{2+} excretion, although, as noted previously, homozygous Nuf mice are affected more severely than the heterozygotes.[123]

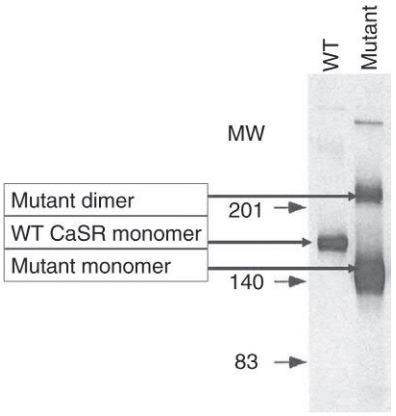

FIGURE 37.5 Relative levels of cell surface expression of wild type and CaSR mutant 895del1075. Cell surface CaSRs were surface labeled by membrane-impermeant biotinylation, solubilized, and then immunoprecipitated. After proteins were separated by polyacrylamide gel electrophoresis of the immunoprecipitate under reducing conditions (to cleave disulfide bonds and increase the amount of monomer), biotin-labeled cell surface CaSR was detected using avidin-horseradish peroxidase, which binds to the biotinylated CaSRs. Note the greater amount of the mutant CaSR, which is of lower molecular weight than the WT receptor because of the deletion in the C-terminus of the mutant CaSR. *Source: Reproduced from Bai M, et al.* J Clin Endocrinol Metab *2000;***85***:1695–1702, with permission.*

There have been no studies to date on the histology of the parathyroid glands in ADH1, although one might assume that they would be present, and potentially might show some sign of structural or functional atrophy in view of their diminished function in vivo. However, as noted earlier, the Nuf mouse, with its activating CaSR mutation has normal parathyroid glands,[123] and the same might be true with humans having ADH. There can be considerable renal pathology related to ADH, much of it iatrogenic and resulting from treatment of the hypoparathyroidism.[118] Presumably as a result of marked hypercalciuria attendant upon the use of oral supplementation with vitamin D metabolites and calcium, there can be renal calcification, nephrocalcinosis, renal stones, and associated impairment of renal function, sometimes irreversible.

Three-dimensional modeling has been used to predict the impact of activating *GNA11* mutations on the G protein's structure and the associated G protein-mediated activation of the CaSR. R181Q and F341L are predicted to modify G protein binding/hydrolysis in ways that would prolong activation of the G protein and, therefore, CaSR-mediated signaling.[36] Similar modeling suggested that the V340M mutation likely alters the conformation of the C-terminal α5 helix, which may affect G protein-coupled receptor binding and G protein activation.[119]

In vitro functional expression of wild type and mutant $G\alpha_{11}$ proteins (V340M, R181Q, and F341L) in HEK293 cells stably expressing the CaSR have demonstrated that the EC_{50}'s for Ca_o^{2+}-elicited increases in Ca_i^{2+} were significantly left shifted compared to the wild-type EC_{50}, consistent with a gain of function.[119] Thus, although the CaSR in patients with ADH2 is intrinsically normal, because of the activated G proteins, less receptor occupancy is required to get a given degree of CaSR-mediated activation of intracellular signaling. As a result, in the parathyroid even overt hypocalcemia is sufficient to suppress PTH secretion to some degree. A further decrease in the serum calcium concentration would be expected to result in stimulation of PTH secretion by reducing the activity of the receptor-G protein complex, although this has not been directly demonstrated. In the kidney, CaSR-mediated activation of the mutant G protein during treatment with calcium and vitamin D would be expected to produce hypercalciuria, as occurs in patients with ADH1 (due to further activation of their mutant CaSRs), even if urine calcium excretion is normal in such patients when untreated.

4.5 Diagnostic Aspects

The diagnosis of ADH1 or 2 should be suspected in any kindred with isolated, autosomal dominant hypocalcemia, or in sporadic cases of idiopathic hypoparathyroidism, particularly those who have had complications, such as marked hypercalciuria and nephrocalcinosis, during treatment with oral calcium and vitamin D metabolites, as noted earlier.[127] The diagnosis should be confirmed by sequencing of the *CaSR* and *GNA11* genes, and affected family members identified by measurement of serum calcium concentration or gene sequencing if needed. Expression studies of the mutant protein can be carried out as appropriate. Currently there are insufficient data regarding the clinical characteristics of patients with ADH2 to sequence one or the other genes first. Renal function should be assessed and urinary calcium excretion measured before and during therapy. Renal ultrasound, plain X-rays or CT should be used, if indicated, to identify and/or monitor renal deposition of calcium and or renal stone disease during treatment.

A small number of patients have been described with functional hypoparathyroidism secondary to activating antibodies to the CaSR rather than irreversible autoimmune destruction of the parathyroid glands.[128] This syndrome should be suspected in patients with hypoparathyroidism associated with other organ specific autoimmunity.

4.6 Counseling

Counseling has an important educational and therapeutic implications related to ADH1 and 2 in two settings. First, affected family members should be well-versed in the rationale for treatment of the condition or

lack thereof, as just described, to minimize the chance of overzealous treatment by the patients' caregivers.[116] Second, gestation of an unaffected fetus in an affected mother could lead to secondary hyperparathyroidism and related complications in the fetus (e.g., hyperparathyroid bone disease), because the fetal parathyroid glands are exposed to the hypocalcemia of the mother. However, while it might be desirable to know the genotype of the fetus prenatally, which has been carried out in one case of ADH1,[109] in practice complications of unaffected neonates born of affected mothers do not appear to be a clinically significant problem. Based on our current state of knowledge, patients with ADH2 can be counseled similarly.

4.7 Treatment

It is important to prevent renal complications (e.g., nephrocalcinosis, nephrolithiasis, and impaired renal function) during treatment of patients with ADH1 or 2 with calcium and vitamin D metabolites, such as calcitriol.[116] Treatment with calcium supplements and vitamin D metabolites should be reserved for those patients with symptomatic ADH; the goal should be to increase the serum calcium concentration only to a level sufficient to render the patient asymptomatic and usually not to a normocalcemic level.[116] Renal excretion of calcium requires monitoring, and it may be necessary to coadminister a hypocalciuric agent, such as a thiazide diuretic or injection or infusion of PTH1-34 or PTH1-84, [129,130] which can lower urinary calcium excretion at any given level of serum calcium. Renal function (e.g., serum creatinine and blood urea nitrogen) should be monitored during treatment. A potential therapy in the future is the use of a CaSR antagonist (calcilytic). This class of drug has been shown to effectively antagonize CaSRs with activating mutations [131] and might "reset" both parathyroid and kidney to maintain more nearly normal calcium homeostasis in both ADH1 and ADH2, which both seem to respond similarly to the calcilytic in vitro.[97] The use of calcilytic might also be helpful in patients with activating antibodies directed at the CaSR.

5 CLINICAL AND GENETIC FEATURES OF BARTTER'S SYNDROME SUBTYPE V ARISING FROM ACTIVATING MUTATIONS OF THE CaSR: [OMIM - #601199.0035]

5.1 Clinical Description

The Bartter–Gitelman spectrum of disorders are a heterogeneous group of syndromes of abnormal renal tubular transport characterized by defects in sodium and chloride reabsorption in the TAL of the loop of Henle (mimicking a furosemide-like effect).[6] One key transporter involved in this spectrum of disorders is the apical Na-K-2Cl (NKCC2) cotransporter. It transports sodium and potassium down an electrochemical gradient into the cell [6] and acts in concert with K^+ recycling via the apical renal outer medullary K^+ channel (ROMK) and basolateral efflux of negatively charged chloride ions through a Cl^- channel.[6] This produces a positive transepithelial potential difference from inside to outside of the TAL that drives the paracellular reabsorption of calcium and magnesium, as well as some sodium, as noted earlier in the section on FHH. The CaSR inhibits the function of the ROMK channel and probably the NKCC2,[6] thereby diminishing the transtubular potential difference and decreasing Ca^{2+} and Mg^{2+} reabsorption in the TAL in the setting of hypercalcemia. The various forms of Bartter's syndrome result from inactivating mutations in the various components making up this transport mechanism, such as, for example, NKCC itself, ROMK or the basolateral Cl^- channel.[132] A more recent addition to the list of causes of Bartter's syndrome is the occasional activating mutation of the CaSR that is thought to produce an exaggerated CaSR-mediated inhibition of tubular cation reabsorption in the TAL, thereby producing Bartter's syndrome subtype V.[133–135] No cases of the Bartter phenotype have been reported to date in ADH2.

5.2 Genetic, Molecular Genetic, Functional, and Molecular Pathology

Within the last 10 years, investigators found activating mutations of the gene for the CaSR in three patients exhibiting features of Bartter's syndrome in addition to those of ADH1, namely L125P, C131W, and A843E.[133,134] The first of these mutations had a greatly reduced EC_{50} for Ca^{2+}, and a marked increase in sensitivity of the mutant CaSRs to Ca^{2+} may be an important common denominator in cases exhibiting the Bartter's phenotype. Another recent case report of this syndrome in monozygotic twins involving the K29E mutation in the ECD of the CaSR described mild hypokalemia, mild abnormalities in aldosterone and renin production, as well as a lack of alkalosis but notable hypocalcemia.[135] The K29E mutation has been reported to be a highly activating mutation of the CaSR (EC_{50} of 1.45 vs. 3.16 mM Ca^{2+}, respectively),[136] and buttresses older observations that the phenotype of Bartter's syndrome is variable and not directly related to the in-vitro potency of the known genetic changes associated with this syndrome.[137] Notably, the identification of the Bartter's phenotype in some cases with activating CaSR mutations further implicates the CaSR not only in calcium and magnesium homeostasis but in salt and water homeostasis.[8]

5.3 Diagnostic Aspects, Counseling, and Treatment

The prominent findings in the Bartter's class of disorders include: renal salt wasting, hypokalemic metabolic alkalosis, elevated renin and aldosterone levels, and normal to low blood pressure.[132] In the small proportion of individuals with Bartter's syndrome subtype V, the additional features of ADH, including hypocalcemia, hyperphosphatemia, hypomagnesemia, relative or absolute hypercalciuria, and low normal or frankly low levels of PTH will provide the key to the diagnosis, and mutational analysis of the CaSR gene should be carried out. Of note, the CaSR-related cases of Bartter's syndrome identified so far have been inherited in an autosomal dominant manner, unlike other subtypes, which are inherited as autosomal recessive traits.

Other forms of Bartter's syndrome can occur in the perinatal period with polyhydramnios and premature delivery or in the first few years of life with polyuria, polydipsia, isosthenuria, or hyposthenuria, failure to thrive and frequent episodes of dehydration.[132] The mainstay of treatment at the present time is nonspecific and includes fluid and electrolyte replacement. Chronic hypokalemia worsens the condition and can be ameliorated with potassium supplementation and/or amiloride or aldosterone antagonists. Magnesium repletion is not infrequently required.

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CHAPTER

38

Multiple Endocrine Neoplasia Syndromes

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1 INTRODUCTION

Multiple endocrine neoplasia (MEN) is characterized by the occurrence of tumors involving two or more endocrine glands within a single patient. Four major forms of MEN are recognized and referred to as MEN types 1–4, and each form is characterized by the development of tumors within specific endocrine tissues (Table 38.1). Each form of MEN is typically inherited as an autosomal-dominant syndrome but may occur sporadically; that is, without a family history, although this distinction between sporadic and familial cases may sometimes be challenging. In addition to MEN1-4, six other syndromes, which are associated with multiple endocrine and other organ neoplasias (MEONs) are recognized (Table 38.2). These include the hyperparathyroidism-jaw tumor (HPT-JT) syndrome, Von Hippel–Lindau (VHL) disease, Carney Complex (CNC), Neurofibromatosis type 1 (NF1), Cowden syndrome (CWD) and McCune–Albright syndrome (MAS). Each of these are typically inherited as autosomal dominant disorders, with the exception of MAS which is due to a mosaic expression of a postzygotic somatic G-protein alpha subunit (*GNAS*) mutation. This chapter focuses on the skeletal diseases associated with MEN and MEONs syndromes.

2 MULTIPLE ENDOCRINE NEOPLASIA TYPE 1

MEN1 is characterized by the combined occurrence of tumors of the parathyroid glands, the pancreatic islet cells, and the anterior pituitary.[1–3] In addition, adrenal cortical tumors, foregut carcinoids, facial angiofibromas, collagenomas, and lipomatous tumors have been described (Table 38.1). MEN1 is a highly penetrant disorder with an estimated incidence of 1:30,000 of the population. Overt clinical manifestations are unusual before the age of 5 years, but thereafter demonstrate an increasing age-related penetrance such that clinical and biochemical manifestations are observed in ~80 and >98% of patients, respectively, by the 5th decade.[4] The clinical manifestations of MEN1 are related to the sites of tumor development and/or respective hormone hypersecretion. Parathyroid tumors are typically the first manifestation of disease although a minority will present with insulinoma, nonfunctioning pancreatic neuroendocrine tumors (P-NETs), or pituitary tumors.[4–7] Occasionally, individuals with MEN1 present with gastrinoma, thymic carcinoid, or adrenal tumors.[6] MEN1-associated tumors are associated with considerable morbidity and mortality, with ~30%–70% of MEN1 individuals dying of causes directly related to MEN1.[8–10] Furthermore, these deaths may occur at a young age with an overall reduced life expectancy and malignant P-NETs and thymic carcinoid tumors are particularly associated with a significant increased in risk of death (hazard ratio >3, $P < 0.005$).[9]

A diagnosis of MEN1 may be established by one of three criteria (Fig. 38.1)[2,11,12]; a clinical diagnosis of MEN1 may be established in an individual in whom two or more MEN1-associated endocrine tumors develop; a familial diagnosis of MEN1 is established for an individual who has the occurrence of one MEN1-associated tumor and is a first degree relative of an individual with a clinical diagnosis of MEN1; and finally, a genetic diagnosis of MEN1 is made by identification of a germline *MEN1* mutation in an individual, who may be asymptomatic and has not yet developed any biochemical or radiological abnormalities indicative of tumor development.[12]

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TABLE 38.1 Multiple Endocrine Neoplasia (MEN) Syndromes and Their Characteristic Tumors and Associated Genetic Abnormalities

Types (chromosome locations)	Tumors (estimated penetrance)	Genes; most frequently mutated codons
MEN1 (11q13)	Parathyroid adenoma (90%)	*MEN1*
	Enteropancreatic tumor (30%–70%)	83/84, 4-bp del (≈4%)
	• Gastrinoma (40%)	119, 3-bp del (≈3%)
	• Insulinoma (10%)	209–211, 4-bp del (≈8%)
	• Nonfunctioning (20%–55%)	418, 3-bp del (≈4%)
	• Glucagonoma (<1%)	514–516, del or ins (≈7%)
	• VIPoma (<1%)	Intron 4 ss, (≈10%)
	Pituitary adenoma (30%–40%)	
	• Prolactinoma (20%)	
	• Somatotrophinoma (10%)	
	• Corticotrophinoma (<5%)	
	• Nonfunctioning (<5%)	
	Associated tumors	
	• Adrenal cortical tumor (20%–40%)	
	• Pheochromocytoma (<1%)	
	• Bronchopulmonary NET (2%)	
	• Thymic NET (2%)	
	• Gastric NET (10%)	
	• Lipomas (30%)	
	• Angiofibromas (85%)	
	• Collagenomas (70%)	
	• Meningiomas (8%)	
MEN2 (10 cen-10q11.2)		
MEN2A	MTC (90%)	*RET*
	Pheochromocytoma (50%)	634, missense, for example, Cys→Arg
	Parathyroid adenoma (20%–30%)	
(MTC only)[a]	MTC (100%)	*RET* 618, missense
MEN2B (also known as MEN3)	MTC (>90%)	*RET* 918, Met→Thr
	Pheochromocytoma (40%–50%)	
	Associated abnormalities (40%–50%)	
	Mucosal neuromas	
	Marfanoid habitus	
	Medullated corneal nerve fibres	
	Megacolon	
MEN4 (12p13)	Parathyroid adenoma[b]	*CDKN1B*

TABLE 38.1 Multiple Endocrine Neoplasia (MEN) Syndromes and Their Characteristic Tumors and Associated Genetic Abnormalities (*cont.*)

Types (chromosome locations)	Tumors (estimated penetrance)	Genes; most frequently mutated codons
	Pituitary adenoma[b]	No common mutations identified
	Reproduction organ tumors[b] (e.g., testicular cancer and neuroendocrine cervical carcinoma)	
	Adrenal + renal tumors[b]	

Autosomal-dominant inheritance of the MEN1 syndrome has been established. del, Deletion; ins, insertion; MTC, medullary thyroid cancer; NET, neuroendocrine test; PPoma, pancreatic polypeptide-secreting tumor; VIPoma, vasoactive intestinal polypeptide-secreting tumor.
[a]*The frequency of the MTC only variants has been reported to be low and recent guidelines suggest it is considered a variant of MEN2A.*
[b]*Insufficient numbers reported to provide prevalence information.*
Adapted from Thakker RV et al. Multiple endocrine neoplasia: syndromes of the twentieth century. J Clin Endocirnol Metabol *1998;***83***:2817–2620, with permission.*

TABLE 38.2 Multiple Endocrine and Other Organ Neoplasias (MEONs) Syndromes and Their Associated Genes and Chromosomal Locations

Diseases[a]		Genes	Chromosomal location
HPT-JT		*CDC73*	1q31.2
Carney complex			
	CNC1	*PRKAR1A*	17q24.2
	CNC2	*Unknown*	2p16
VHL		*VHL*	3p25
NF1		*NF1*	17q11.2
CWS			
	CWS1	*PTEN*	10q23.31
	CWS2	*SDHB*	1p36.13
	CWS3	*SDHD*	11q23.1
	CWS4	*KLLN*	10q23.31
	CWS5	*PIK3CA*	3q26.32
	CWS6	*AKT1*	14q32.33
	CWS7	*SEC23B*	20p11.23
MAS		*GNAS*	20q13.32

CNC, Carney complex; CWS, Cowden Syndrome; HPT-JT, Hyperparathyroidism-Jaw Tumor; MAS, McCune Albright syndrome; NF1, Neurofibromatosis type 1; VHL, Von Hippel–Lindau disease.
[a]*The inheritance for these disorders is autosomal dominant, except MAS, which is due to mosaicism that results from the postzygotic somatic cell mutation of the* GNAS *gene, encoding Gαs.*
Adapted from Thakker RV. Multiple endocrine neoplasia. In: Kasper et al. editor. Harrison's Principles of Internal Medicine. *19th ed. McGraw-Hill Education; 2015, with permission.*

2.1 Parathyroid Tumors

2.1.1 Clinical Findings

Primary hyperparathyroidism is the most common feature of MEN1 and occurs in approximately 95% of all patients with MEN1.[4,7] Patients may have asymptomatic hypercalcemia, or symptoms associated with hypercalcemia (e.g., polyuria, polydipsia, constipation, or malaise), as well as nephrolithiasis, osteitis fibrosa cystica, or peptic ulcers. Biochemical investigations reveal hypercalcemia, usually in association with raised circulating parathyroid hormone (PTH) concentrations. The hypercalcemia is usually mild, and severe hypercalcemia is a rare occurrence. Parathyroid carcinoma is extremely rare in the setting of MEN1. Additional differences in the primary hyperparathyroidism of patients with MEN1, as opposed to those with sporadic parathyroid disease, include an earlier age of onset (20–25 vs. 55 years) and an equal male/female ratio (1:1 vs. 1:3).[1,4,13,14] In addition, MEN1-associated primary hyperparathyroidism is typically associated with synchronous or asynchronous involvement of all four glands. Symptomatic primary hyperparathyroidism in patients with MEN1 is unusual before the age of 15 years although biochemical evidence of primary hyperparathyroidism may often be identified before this age and has been reported in those <5 years.[6]

2.1.2 Treatment

Surgical removal of the abnormally overactive parathyroids is the definitive treatment, but it is controversial whether to perform subtotal (i.e., removal of 3.5 glands) or total parathyroidectomy with or without autotransplantation of parathyroid tissue, and whether it should be performed at an early or late stage.[2,15–17] Given expected multigland involvement, preoperative localization studies are typically considered of limited use, with the need for 4-gland exploration at the time of surgery. While total parathyroidectomy is reported to be associated with an increased rate of biochemical cure, it is also associated with an increased rate of long-term hypoparathyroidism. Subtotal parathyroidectomy (with removal of ≥3.5 glands) may be associated with modestly lower rates of biochemical cure but lower rates of permanent hypoparathyoridism.[15,18] Concurrent transcervical thymectomy is frequently performed; potential benefits include an increased likelihood of identifying (and removing) accessary/supernumerary parathyroid tissue, as well as reducing the risk of thymic carcinoids,[2,15,17,19] although transcervical thymectomy does not entirely eliminate the risk of thymic carcinoid.[20] Minimally

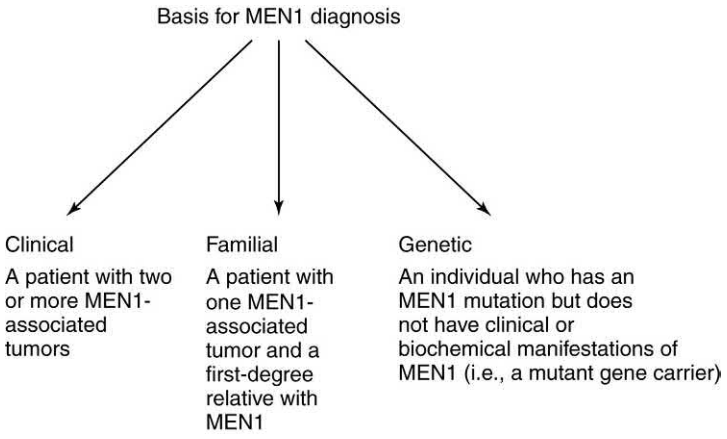


FIGURE 38.1 Basis for a diagnosis of multiple endocrine neoplasia type1 (MEN1) in individuals. A diagnosis of MEN1 based on clinical and familial criteria may be confounded by the occurrence of phenocopies. *Source: Reproduced from Turner JJ, Christie PT, Pearce SH, Turnpenny PD, Thakker RV. Diagnostic challenges due to phenocopies: lessons from multiple endocrine neoplasia type1 (MEN1).* Hum Mutat *2010;***31***(1):E1089–E1101, with permission.*

invasive parathyroidectomy is not recommended,[2] although some investigators have advocated a unilateral surgical approach in those with disease limited to a single side, in the knowledge that surgery on the contralateral side will likely be required at a later stage.[21] Given the different approaches advocated, the type and timing of surgery requires careful consideration, and factors, such as surgical experience, availability of vitamin D analogs for subsequent treatment of long-term hypoparathyroidism, and the preferences of the patient should be considered. Calcimimetics (e.g., cinacalcet) have been used to treat primary hyperparathyroidism in some patients in whom surgery had failed or was contraindicated.

2.2 MEN1-Associated Primary Hyperparathyroidism and Relevance to Skeletal Disease

Observational studies have reported that MEN1-associated primary hyperparathyroidism is associated with a greater reduction in bone mineral density (BMD) than those with sporadic disease.[14,22–24] Indeed, osteoporosis and osteopenia are frequently observed in affected individuals.[22–24] Specifically, greater reductions in BMD at the lumbar spine, femoral neck, and distal radius have been observed in MEN1-associated primary hyperparathyroidism than in those with equivalent sporadic disease with greater degrees of bone demineralization.[23,24] However, it is unclear whether such features are associated with the chronicity of hyperparathyroidism or represent a genuine difference in disease pathogenesis. The effects of surgery on BMD have also been assessed. Following parathyroid surgery marked and significant improvements in BMD have been reported in the lumbar spine and femoral neck, with minimal or only modest effects observed on the distal radius.[25] Further large prospective studies are required to determine whether surgical intervention is associated with long-term improvement in bone health and clinical outcomes.

2.3 Pancreatic Tumors

The incidence of clinically relevant gastro-P-NETs in patients with MEN1 varies from 30% to 80% in different series (Table 38.3).[7,26,27] However, microscopic islet abnormalities are reported with very high prevalence,[28,29] while improved imaging modalities, including endoscopic ultrasound indicate a high prevalence of subclinical disease.[26,30] Tumors (Table 38.3) may secrete excessive levels of specific hormones [e.g., gastrin, insulin, glucagon, or vasoactive intestinal polypeptide (VIP)], in association with distinct clinical syndromes, or may be classified as nonfunctional due to the absence of excess hormone production and/or secretion. It is important to recognize that individuals with MEN1 may have multiple tumors that are hormonally and pathologically distinct. An overview of a suggested clinical, investigative, and management approach to pancreatic NETs in patients with MEN1 is provided in Fig. 38.2.

2.3.1 Gastrinoma

2.3.1.1 Clinical Findings

Gastrin-secreting tumors (gastrinomas) are associated with marked gastric acid production, and recurrent peptic ulceration, a combination also referred to as the Zollinger–Ellison syndrome. Patients with Zollinger–Ellison syndrome may also suffer from diarrhea and steatorrhea. Gastrinomas occur in 30%–40% of patients with MEN1 and typically occur in those older than 40 years.[1,4,26,31] In contrast to sporadic gastrinomas, which mostly arise within the pancreas, the majority of MEN1-associated gastrinomas are microscopic and located in the duodenal mucosa.[32,33] The morbidity associated with gastrinomas in individuals with MEN1 following the

TABLE 38.3 Characteristics of MEN1-Associated Pancreatic Neuroendocrine Tumors

Tumor subtypes	Hormones produced	Clinical presentations	Mean size (cm)	Metastatic sites (%)	Mean prevalences (%)	Important differences versus non-MEN1-associated P-NETs
Nonfunctioning	May produce hormones that are not secreted, clinically inert, or inadequately concentrated to induce symptoms	Incidental finding on imaging/biopsy; direct tumor mass effects	<1.0	LN (14–33)[a]; liver (9–19)[a]	96% (Range 80–100) (0%–13% large/ symptomatic)	Small size, multiplicity, background of diffuse pancreatic microadenomatosis
Gastrinoma	Gastrin	Abdominal pain, heartburn, nausea, gastrointestinal bleeding diarrhea (steatorrhea)	<1.0	LN (34–85); liver (2–14)	54% (Range 20–61)	Younger age of presentation, small size, >80% located in duodenum, multiplicity, background of diffuse pancreatic microadenomatosis, early occurrence of LN metastases, and low likelihood of surgical cure
Insulinoma	Insulin	Neuroglycopenia, hypoglycemia, catecholaminergic symptoms	<1.0	LN (0–10); liver (0–20)	18% (Range 7–31)	Younger age of presentation, background of diffuse pancreatic microadenomatosis
Rare functioning	Glucagon	(?)[b]	Median 3.3 (range 0.9–11.0)	Lymph node (25); liver (40)[c]	3% (Range 1–6)	Background of diffuse pancreatic microadenomatosis
Rare functioning	Vasoactive intestinal peptide	Severe watery diarrhea, hypokalemia, achlorhydria	Median 4.0 (range 3–60)	LN (0–100); liver (0–33)[c]	3% (Range 1–12)	Background of diffuse pancreatic microadenomatosis
Rare functioning	Somatostatinoma	(?)[d]	6.0 and 8.0	LN (50); lung (50)[c]	<1%	Background of diffuse pancreatic microadenomatosis
Rare functioning	Other: growth hormone releasing hormone; adrenocorticotrophic hormone; PTH related peptide; carcinoid	Variable			<1%	Background of diffuse pancreatic microadenomatosis

LN, Lymph node.

[a]*These data report metastasis rates from macroscopic tumors, that is, >2 cm in size. There are limited data from tumors <1cm and these suggest a low metastasis rate of ~4%.*

[b]*The classical signs of the glucagonoma syndrome: necrolytic erythema, thromboembolism, and weight loss have not been observed in MEN1 patients.*

[c]*Very low sample sizes for rare functioning P-NETs of between 1 and 5 cases.*

[d]*Somatostatinomas are found in MEN, but have not been reported to be associated with signs of the somatostatinoma syndrome: steatorrhea, diarrhea, cholelithasis, and weight loss in MEN1 patients.*

Adapted from Yates CJ, Newey PJ, Thakker RV. Challenges and controversies in management of pancreatic neuroendocrine tumours in patients with MEN1. Lancet Diabetes Endocrinol *2015;**3**:895–905, with permission.*

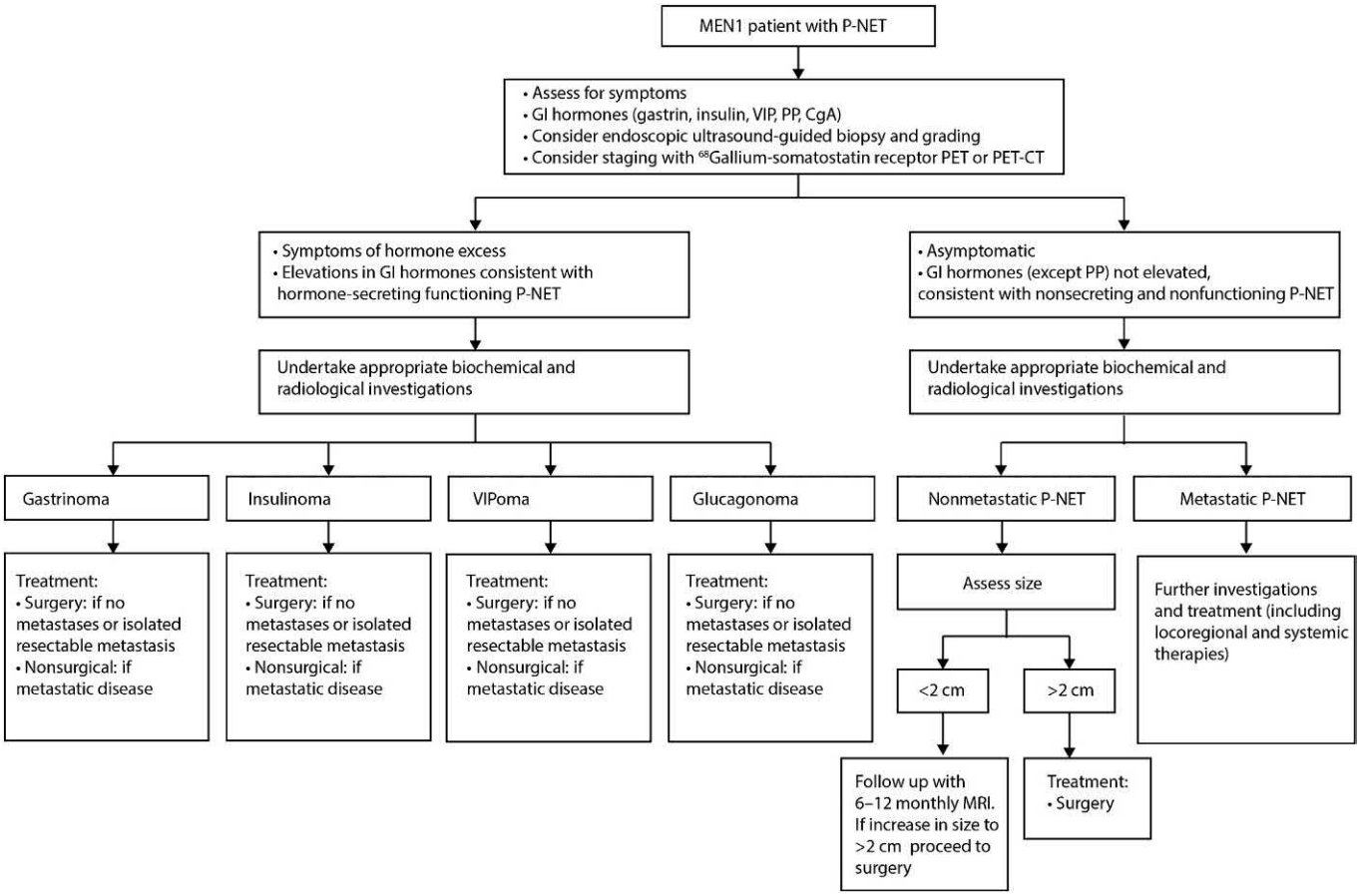


FIGURE 38.2 Suggested clinical, investigative, and management approach to MEN1 patients with pancreatic neuroendocrine tumors (P-NETs). All investigations and management plans should be discussed at a multidisciplinary team meeting. The use of endoscopic ultrasound-guided biopsy and grading, and staging with 68 gallium–somatostatin receptor PET/CT, would represent incorporation of recent developments into the reported clinical guidelines. *Source: Adapted from Yates CJ, Newey PJ, Thakker RV. Challenges and controversies in management of pancreatic neuroendocrine tumors in patients with MEN1.* Lancet Diabetes Endocrinol *2015;**3**(11):895–905, with permission.*

introduction of acid-suppressive therapies (i.e., proton pump inhibitors and histamine H2-receptor antagonists) has shifted from that associated with severe peptic ulceration, hemorrhage, and >80% mortality to that associated with the consequences of malignant disease. Indeed, MEN1 gastrinomas frequently have metastasized to local lymph nodes prior to the diagnosis being established.[3,32]

2.3.1.2 Treatment

Medical treatment of patients with MEN1 and Zollinger–Ellison syndrome is directed toward reducing basal acid secretion typically employing parietal cell H^+-K^+-adenosine triphosphatase (ATPase) inhibitors (e.g., omeprazole or lansoprazole) or histamine H_2 receptor antagonists (e.g., ranitidine).[2] The role of surgery in the treatment of gastrinomas in patients with MEN1 is controversial. While surgery is indicated for those rare patients with a single pancreatic gastrinoma >2–2.5 cm, its role in duodenal gastrinomas is less established, particularly as affected individuals may have a good long-term prognosis with medical therapy. A number of surgical approaches have been advocated for duodenal gastrinoma which demonstrate postoperative eugastrinemia, although long-term outcome data is required to determine their clinical utility.[3,26,33] Treatment of disseminated gastrinomas is difficult, with chemotherapy, hepatic artery embolization, and debulking surgery having occasional success.

2.3.2 *Insulinoma*

2.3.2.1 Clinical Findings

Insulinomas occur in 10%–30% of individuals with MEN1 and typically affect those who are <40 years of age (Table 38.3), including children and young adults.[6] Patients with insulinoma present with hypoglycemic symptoms that develop after a fast or exertion and improve after glucose intake. Diagnosis of insulinoma requires the demonstration of hypoglycemia (e.g., plasma glucose <2.2 mmol) in association with high or inappropriately normal concentrations of insulin, C-peptide, and proinsulin.[2] Careful preoperative localization is required to identify the likely source of insulin hypersecretion, and may include endoscopic ultrasonography, CT

or MRI scanning, selective intraarterial stimulation with hepatic venous sampling, and intraoperative direct pancreatic ultrasonography.

2.3.2.2 Treatment

Surgical treatment is indicated for patients with operable tumors.[34] Medical treatment, which consists of frequent carbohydrate meals and diazoxide or octreotide, may be required preoperatively or in patients with inoperable disease. Chemotherapy consisting of streptozotocin, 5-flurouracil, and doxorubicin, or hepatic artery embolization has been used for metastatic disease.

2.3.3 Glucagonoma

2.3.3.1 Clinical Findings

Glucagonomas, glucagon-secreting pancreatic tumors, occur in fewer than 3% of patients with MEN1.[9,35] The characteristic clinical manifestations of a skin rash (necrolytic migratory erythema), weight loss, anemia, and stomatitis may be absent.

2.3.3.2 Treatment

Surgical removal of the glucagonoma is the treatment of choice. However, treatment may be difficult because approximately 50%–80% of patients have metastases at the time of diagnosis. Medical treatment with somatostatin analogs (e.g., octreotide or lanreotide), or chemotherapy has been successful in some patients, and hepatic artery embolization has been used to treat metastatic disease.

2.3.4 Vasoactive Intestinal Peptide (VIP) Tumors (VIPomas)

2.3.4.1 Clinical Findings

Vasoactive intestinal peptide tumors (VIPomas) have been reported in only a few patients with MEN1, who develop watery diarrhea, hypokalemia, and achlorhydria.[9,35] The diagnosis is established by confirming a high stool volume during a fast together with markedly increased plasma VIP concentrations.

2.3.4.2 Treatment

Surgical management of VIPomas, which are mostly located in the tail of the pancreas, has been curative. However, in patients with unresectable tumor, treatment with somatostatin analogs, chemotherapy, corticosteroids, indomethacin, metoclopramide, and lithium carbonate has proved beneficial, and hepatic artery embolization has been useful for the treatment of metastases.

2.3.5 Other Rare Functioning Pancreatic NETs

NETs secreting growth hormone (GH)-releasing hormone, growth hormone tumors, have been reported occasionally in MEN1, as have tumors secreting somatostatin although these are not typically associated with the "somatostatinoma syndrome" consisting of hyperglycemia, cholelithiasis, low acid output, steatorrhea, diarrhea, abdominal pain, anemia, and weight loss.

2.3.6 Nonfunctioning Pancreatic Neuroendocrine Tumors

2.3.6.1 Clinical Findings

Nonfunctioning P-NETs are either nonsecretory, or secrete pancreatic polypeptide (PP) (or other hormones) that is not associated with symptoms. Nonfunctioning P-NETs are now recognized as the most common P-NET in MEN1 with clinically relevant disease (e.g., macroscopic tumors) occurring in 30%–60% of individuals, and are found by histology in >95% of surgically removed pancreatic samples.[26–28,36] Nonfunctioning P-NETs are most common in those >20 years, but they can also occur in asymptomatic individuals <15 years of age.[5]

The optimum biochemical and radiological modalities for diagnosis remain to be established. For example, existing biomarkers, including glucagon, PP, and chromogranin A have low sensitivities and specificities.[26,37,38] Endoscopic ultrasound likely represents the most sensitive imaging modality for the detection of small P-NETs, while somatostatin receptor scintography is the most reliable method for detecting metastatic disease. However, cross sectional imaging with MRI is often recommended as first line investigation.[2]

2.3.6.2 Treatment

Nonfunctioning P-NETs represent the leading cause of death in individuals with MEN1, although their management remains controversial.[26,27,39] The risk of liver metastases is reported to correlate with tumor size and most centers recommend surgery for tumors >2 cm, although current guidelines recommend consideration of surgical resection for tumors >1 cm.[2] A number of therapies are available for those with advanced disease. Systemic therapies include somatostatin analogues, chemotherapy, tyrosine kinase inhibitors (TKIs), mammalian target of rapamycin (mTOR) inhibitors, and peptide receptor radionuclide therapy. Locoregional therapies include cytoreductive surgery, radiofrequency ablation, transarterial embolization/chemoembolization, and selective internal radiation therapy.[2,26]

2.4 MEN1-Associated Pancreatic NETs and Relevance to Skeletal Disease

The direct relevance of P-NETs to bone health is predominantly limited to the risk of skeletal metastases. Studies evaluating the frequency of skeletal metastases of MEN1-associated pancreatic NETs are lacking,

although evaluation of the sporadic counterparts indicates that bone involvement is uncommon. For example, observational studies indicate that skeletal metastases are present in 5%–10% of individuals with gastrinoma, and typically only occur in the presence of hepatic involvement, while they are infrequently observed in those with insulinoma, glucagonoma, and nonfunctioning tumors.[40–42] In addition to the direct involvement of bone in advanced P-NETs, mechanisms for secondary skeletal involvement are evident. For example, P-NETs and gastrinoma may rarely be associated with ectopic ACTH secretion with the potential for secondary osteoporosis due to hypercortisolemia[43,44]. In addition, individuals with microscopic duodenal gastrinomas are frequently treated with long-term PPI therapy, and recent studies have highlighted that PPIs may impact on bone health, including a reduction in BMD, although the mechanisms leading to such an effect remain ill defined.[45] Finally, individuals undergoing extensive pancreatic resection, may be at risk of malabsorption and/or pancreatic insufficiency with development of vitamin D deficiency, osteomalacia, and osteoporosis, although this has not been formally evaluated in the context of MEN1. The presence of comorbid MEN1-associated conditions (e.g., primary hyperparathyroidism) may also influence the likelihood and/or severity of skeletal manifestations.

2.5 Pituitary Tumors

2.5.1 Clinical Findings

The incidence of pituitary tumors in patients with MEN1 varies from 15% to 90% in different series, and about two-thirds of these are microadenomas (diameter <1 cm).[4,46,47] Approximately 60% of MEN1-associated pituitary tumors secrete prolactin (PRL), fewer than 25% secrete GH, 5% secrete adrenocorticotrophic hormone, and the remainder appear to be nonfunctioning. The clinical manifestations of these tumors depend on the impact on hormone secretion and the size of the pituitary tumor.[46] Thus, patients may have symptoms of hyperprolactinemia (e.g., amenorrhea, infertility, and galactorrhea in women and impotence in men), acromegaly, or Cushing's disease. Large pituitary tumors may compress adjacent structures, such as the optic chiasm or normal pituitary tissue and cause bitemporal hemianopia or hypopituitarism, respectively.

2.5.2 Treatment

Treatment of pituitary tumors in patients with MEN1 is similar to that in patients without MEN1 and consists of appropriate medical therapy (e.g., bromocriptine or cabergoline for prolactinoma; or octreotide or lanreotide for somatotrophinoma) or selective hypophysectomy by the transsphenoidal approach if feasible, with radiotherapy being reserved for residual unresectable tumor.

2.6 MEN1-Associated Pituitary Tumors and Relevance to Skeletal Disease

Each of the main classes of functioning pituitary tumor observed in MEN1, including somatotrophinoma, prolactinoma, and corticotrophinoma have the potential to significantly impact upon bone health. In addition, individuals with hypopituitarism either as a direct result of a pituitary tumor, or due to its treatment (i.e., surgery and radiotherapy) may manifest a skeletal phenotype.

2.6.1 Prolactinoma

The direct impact of PRL excess on bone health is frequently confounded by coexistent secondary hypogonadism. Indeed, the majority of skeletal effects observed in individuals with prolactinomas are ascribed to associated hypogonadism.[48–51] Several studies have demonstrated reductions in BMD affecting the spine, forearm, and hip in individuals with hyperprolactinemia, while individuals with untreated prolactinomas are reported to have an increased risk of fracture.[48–50,52] Importantly, normalization of PRL with restoration of gonadal function is associated with improvements in bone mass, although BMD may not be restored to normal levels.[50] PRL may mediate direct effects on bone as the PRL receptor (Prlr) is expressed in murine osteoblasts, while homozygote *Prlr* knockout mice ($Prlr^{-/-}$) demonstrate reduced bone mass and altered bone development in late embryonic development.[53,54]

2.6.2 Somatotrophinomas and Growth Hormone

GH action on the liver promotes production of insulin-like growth factor (IGF1) and both have a number of direct influences on bone and cartilage function, acting either independently or through a complex interplay. For example, the growth hormone receptor (GHR) is expressed in both osteoblasts and chondrocytes, with GH activity resulting in proliferation of the osteoblast lineage, while synergistic activity with IGF-1 mediate several of its anabolic effects.[55,56] GH is also reported to be more widely involved in mesenchymal precursor cell differentiation, promoting chondrogenesis and osteoblastogenesis, and inhibiting adipogenesis, with subsequent involvement in mature osteoblast function.[55,56] GH is also involved in longitudinal bone growth and bone remodeling.

Unsurprisingly, disorders of GH/IGF-1 are associated with bone and joint disease.[56] Thus, individuals with acromegaly have increased skeletal fragility and several studies have indicated an increased risk of vertebral fracture.[56–58] In addition, reduced cortical and trabecular bone are reported in the proximal femur. However, it is noteworthy that measurement of BMD may not provide an accurate measure of bone health in acromegaly due to underlying abnormalities of bone architecture, as well as

potential for bone enlargement and osteophyte formation. Individuals with acromegaly are also at risk of arthropathy, with features similar to degenerative primary osteoarthritis.[56] The priority in treating acromegalic bone disease involves biochemical control of GH/IGF-1 levels although joint disease may persist and/or progress even when biochemical cure has been achieved. GH deficiency, typically observed in the context of hypopituitarism, may also be associated with reduced BMD and an increased risk of fracture.[59] Sustained GH replacement therapy (i.e., >12 months) has been reported to result in improvement in lumbar spine and femoral neck BMD although further studies are required to determine systematic outcomes on fracture risk.[60]

2.6.3 Corticotrophinomas and Glucocorticoid Excess

Cushing's disease associated with corticotrophinomas, and Cushing's syndrome due to glucocorticoid excess associated with adrenal tumors, lead to osteopenia and osteoporosis, as well as defects in bone microarchitecture, which also contribute to increased fracture risk.[61,62] In addition to direct glucocorticoid activity on bone, secondary effects may arise from associated hypogonadism, altered adrenal androgen production, and changes in insulin and GH signaling.[62] The principal changes in bone metabolism related to glucocorticoid excess include decreased bone formation and impaired osteoblast function. Glucocorticoids inhibit osteoblastogenic differentiation, while promoting apoptosis of both osteoblasts and osteoclasts, thereby reducing both new bone formation and bone remodeling.[62] The consistent observation of reduced bone formation, altered bone microarchitecture, and increased skeletal fragility in individuals with Cushing's syndrome is consistent with increased fracture risk. Symptomatic and asymptomatic thoracic and lumbar vertebral fractures are most frequently observed, while low trauma fractures of the hip, ribs, and pelvis are also commonly observed. Biochemical cure of Cushing's syndrome is typically associated with a marked recovery of BMD, although the requirement for postoperative hydrocortisone treatment may impact upon this.

2.7 Carcinoid Tumors

2.7.1 Clinical Findings

Thymic and bronchial carcinoid tumors occur in 2%–8% of patients with MEN1.[31,63–66] While MEN1-associated bronchial carcinoids were initially reported to occur predominantly in women, the incidence appears to be approximately equal between males and females. In contrast, thymic carcinoids occur predominantly in men, and have an aggressive course with a 10-year survival of ~30%–35%.[9,31,64] As a consequence, interval surveillance imaging with CT or MRI is recommended for their early detection, although the value of such surveillance has not been evaluated.[2] Many patients with thymic and bronchial carcinoids are asymptomatic and do not typically have the symptoms of carcinoid syndrome. Similarly, elevations of plasma Chromogranin A and B, or urinary 5-hydroxyindoleacetic acid levels are not typically observed. In addition to bronchial and thymic carcinoids, gastric carcinoids are observed in individuals with MEN1. Typically, these are of the type II gastric enterochromaffin-like cell carcinoids (ECLomas) and are frequently detected incidentally at the time of gastric endoscopy for dyspeptic symptoms.[67] Tumors are usually multiple and smaller than 1.5 cm.

2.7.2 Treatment

Surgical removal of thymic and bronchial carcinoids, where possible, is the treatment of choice. For unresectable thymic tumors and metastatic disease, somatostatin analogs, systemic chemotherapy, radiotherapy, or peptide receptor radionuclide therapy, may be considered although none have been systematically evaluated in this setting. For gastric carcinoids, surgical resection is frequently recommended, as these tumors may be associated with angioinvasion and local lymph node metastases. Somatostatin analog therapy has been used for gastric carcinoids and resulted in improvements in symptoms and regression of some tumors.

2.8 MEN1-Associated Carcinoid Tumors and Relevance to Skeletal Disease

Skeletal metastases are observed in ~20%–35% of individuals with thymic carcinoid in MEN1.[63,64,66] MRI is the imaging modality of choice for early detection of skeletal disease, with isotope bone scans being also of value. An indirect consequence of carcinoid tumors on bone health may arise from the development of ectopic tumoral ACTH secretion and the development of Cushing's syndrome (see earlier).[43,44,68]

2.9 Adrenocortical Tumors

2.9.1 Clinical Findings

The incidence of asymptomatic adrenocortical tumors in patients with MEN1 has been reported to be as high as 40%. An analysis of >700 MEN1 cases identified adrenal enlargement in ~20% of MEN1 patients with >50% of these individuals harboring tumors >10 mm in size.[69] However, hormonal hypersecretion is uncommon and occurs in ~15% of patients with tumors >10 mm. Of the functioning tumors, primary hyperaldosteronism (Conn's syndrome) and ACTH-independent Cushing's syndrome are most frequently reported, with pheochromocytoma occurring rarely.[69] Adrenocortical carcinoma is rare in MEN1, but should be considered in all individuals with adrenal enlargement which lack imaging characteristics of a benign adenoma.

2.9.2 *Treatment*

In patients with adrenal enlargement without hormonal hypersecretion, and benign imaging characteristics, surveillance is recommended.[69] Surgery should be considered for nonfunctioning adrenal tumors >40 mm in diameter, and for those with atypical radiological features irrespective of size (e.g., increased unenhanced attenuation values and/or delayed contrast washout). The treatment of functioning adrenal tumors in MEN1 is similar to that for sporadic tumors with surgical resection the approach of choice.

2.10 MEN1-Associated Adrenal Tumors and Relevance to Skeletal Disease

The potential for skeletal involvement depends on the subtype of adrenal tumor. Cortisol-secreting adrenal adenomas (or carcinomas) giving rise to ACTH-independent Cushing's syndrome are associated with features of glucocorticoid excess (see earlier). Skeletal metastases from adrenocortical carcinoma and malignant pheochromocytoma are reported in advanced forms of sporadic disease and are likely to occur in MEN1 patients, although their infrequent occurrence in MEN1 precludes any estimate of their frequency of occurrence.

2.11 Other Clinical Manifestations of MEN1

Subcutaneous lipomas, facial angiofibromas, and collagenomas are common in MEN1 patients. Thyroid abnormalities, including adenomas, colloid goiters, and carcinomas, have been reported in MEN1 patients although the high prevalence of thyroid disorders in the general population may confound any genuine association. Recently, an increased risk of breast cancer has been reported in female individuals with MEN1. In independent cohorts, an increased relative breast cancer risk of ~2.3–2.8 was also observed and additional studies are awaited to confirm this finding.[70]

2.12 *MEN1* Gene Function and Clinical Applications

The *MEN1* gene, located on chromosome 11q13, consists of 10 exons that encode a 610-amino acid protein, referred to as menin.[71] To date >1500 germline and somatic *MEN1* mutations have been reported, and the majority (~75%) are nonsense, frameshift, and splice-site mutations that are predicted to result in a truncated protein, ~20%–25% are missense mutations, and ~1% are whole or partial gene deletions.[71,72] More than 90% of tumors from MEN1 patients have loss of heterozygosity at the 11q13 locus, and this has generally been taken as evidence that the *MEN1* gene acts as a tumor-suppressor gene.[71] Approximately 10% of the *MEN1* mutations arise de novo, while ~5%–10% of patients with MEN1 do not appear to harbor mutations in the *MEN1* coding region, and these individuals may harbor mutations in the promoter or untranslated regions, or represent phenocopies or have mutations in other genes, for example, *cyclin-dependent kinase inhibitor 1B* (*CDKN1B*) causing MEN4.[71] Genotype–phenotype correlations are not observed in MEN1. However, germline *MEN1* mutations have been reported in kindreds with familial isolated hyperparathyroidism (FIHP), characterized by the sole occurrence of parathyroid tumors, although this classification requires caution and individuals should be considered at risk of developing MEN1-associated tumors.[71] Another hereditary variant of MEN1 described as the Burin or prolactinoma variant is reported in individuals with specific nonsense mutations (Tyr312Stop and Arg460Stop) and characterized by a high occurrence of prolactinomas and a low occurrence of gastrinomas. Somatic *MEN1* mutations are observed in the sporadic counterparts of MEN1-associated tumors at different frequencies.[71] For example, ~20%–40% of parathyroid tumors, ~40% of gastrinomas, and ~40% of P-NETs harbor somatic *MEN1* mutations often with chromosome 11q13 loss of heterozygosity, consistent with the proposed tumor suppressor function.

2.13 Function of MEN1 Protein (Menin) and Relevance to Bone Biology

Menin is a ubiquitously expressed, predominantly nuclear protein, with a large number of interacting partners (Table 38.4). Within the nucleus it is reported to act as a scaffold protein regulating many cellular processes, including activation and repression of gene transcription, modulation of cellular signaling cascades, and maintenance of the cell cycle, cell integrity, and DNA repair (Table 38.4).[73] The recent resolution of the crystal structure of Menin both in isolation and in complex with key interacting peptides, and the development of small molecule inhibitors targeting key protein-protein interactions has significantly advanced the field.[74,75] For example, a pivotal role in the regulation of gene transcription arises through Menin's interaction with members of the mixed lineage leukemia and trithorax protein family (Table 38.4). Such interaction facilitates trimethylation of lysine 4 residue of histone H3 (H3K4), a chromatin mark linked to activation of transcription.[76–78] It is increasing apparent that Menin activity is both cell-type and context-dependent, and may harbor both tumor suppressor and oncogenic activities. For example, recent studies indicate that Menin influences the outcome of downstream KRAS signaling pathways in a cell-context dependent manner, consistent with its tumor suppressor function in endocrine tissues.[79] In contrast, a protumorigenic function of Menin was recently reported in studies modeling glioma formation in which a potent inhibitor of the Menin-mixed lineage leukemia interaction was identified as the leading "hit" in a drug screen to prevent tumor

TABLE 38.4 Functions of Menin Indicated by Direct Interactions With Proteins and Other Molecules

Functions	Interacting partners
Transcription regulation	JUND
	NF-κB (p50, p52, and p65)
	PEM
	SIN3A
	HDAC
	SMAD1
	SMAD3
	SMAD5
	RUNX2
	MLL histone methyl–transferase complex
	ERα
	CHES1
	Double-stranded DNA
Genome stability	RPA2
	FANCD2
Cell division	NMMHC II-A
	GFAP
	Vimentin
Cell cycle control	NM23 (A)
	ASK

Menin has a large number of reported interacting protein partners. These interactions are involved in several key cellular functions, including activation and repression of transcription, cell-cycle control, alterations in cell motility, cell differentiation, apoptosis, and DNA repair. The Menin-MLL interaction is required for regulation of histone methyltransferase activity, and determines the transcription of key genes, including the cyclin-dependent kinase inhibitors (CDKIs) p27 and p18, and several members of the homeobox (*Hox)* gene family. The interaction with MLL is also responsible for Menin's role as an oncogenic cofactor in acute myeloid leukemia, whereas blockade of the Menin-MLL interaction using small molecule inhibitors may inhibit the proliferation of leukemic cells. Menin also acts to repress gene transcription. For example, the interaction with the activating protein-1 (AP-1) transcription factors (JunD), results in repression of JunD-mediated transcription via recruitment of an mSin3A-histone deacetylase (HDAC) complex. Recently, the forkhead transcription factor CHES1 has been shown to be a component of the transcriptional repressor complex and through an interaction with Menin, regulates the S-phase checkpoint pathway related to DNA damage response. Additional interactions include those with EZH2, a histone methyltransferase forming part of the polycomb repressive complex 2 (PRC2), which is associated with H3K27 methylation and gene repression.
Adapted from Lemos M, Thakker RV. Multiple endocrine neoplasia type 1 (MEN1): analysis of 1336 mutations reported in the first decade following identification of the gene. Hum Mutat *2008;***29***: 22–32, with permission.*

formation.[80] Similar oncogenic functions are reported in leukemia and prostate cancer. Menin is also reported to influence several additional signaling cascades, including the transforming growth factor-β (TGFβ), bone morphogenic protein (BMP), hedgehog, Akt and Int/Wingless (Wnt) pathways.[73,81–83] For example, recent studies indicate that Wnt activation may represent a key step in MEN1-associated pancreatic neuroendocrine tumorigenesis.[83]

Insights into the in vivo function of menin have arisen through the generation of several conventional and conditional Men1 mouse models. Conventional *Men1* knockout models show many similarities to the human disease; heterozygous ($Men1^{+/-}$) mice develop multiple tumors, including those of the pancreas, anterior pituitary, parathyroids, and adrenal tumors that are typically found in MEN1 patients. However, $Men1^{+/-}$ mice also develop gonadal and thyroid tumors, that are not typically associated with MEN1.[84,85] Homozygous knockout ($Men1^{-/-}$) mice demonstrate embryonic lethality with craniofacial defects, hemorrhages, edema, and neural tube defects, although the timing of death and specific phenotype observed is dependent on the background strain of mouse indicating a possible role for genetic modifiers in menin-dependent developmental processes.[84,86] Pancreatic β-cell-specific ablation of *Men1* results in early onset pancreatic islet cell hyperplasia, followed by insulinoma formation,[87] while targeted deletion of *Men1* in both endocrine and exocrine pancreatic cells (i.e., employing a Pdx1-Cre promoter) also displays increased endocrine cell proliferation and tumor formation.[88] Notably, pancreatic α-cell-specific *Men1* ablation, results in insulinoma formation in older mice indicating possible transdifferentiation of α-cells to β-cells, or important paracrine effects.[89,90] Murine *Men1* models have also been used to establish proof of principle for the evaluation of novel therapeutic approaches, including *Men1* gene therapy and pasireotide treatment.[91,92]

The observation that *Men1*-null mice die in midgestation and harbor craniofacial defects indicates that menin may have a role in endochondral and intramembranous bone formation.[84,93] Several studies have highlighted key roles for menin in both osteoblast differentiation and determination of bone mass.[93] Thus, menin is required for the commitment of multipotent mesenchymal stem cells to an osteoblast lineage. This commitment involves menin regulating bone morphogenic protein-2 signaling via members of the Smad family, as well as the key osteoblast transcriptional regulator, Runx2.[81] Once osteoblast commitment is determined, the function of menin function changes to that of inhibiting the later stages of osteoblast differentiation and maturation, which partly occurs by an interaction with the transcription factor JunD.[94] Studies of bone specific conditional *Men1* mouse models have also revealed other roles for menin in bone development. Thus, tissue-specific inactivation of menin in Pax3 or Wnt-1 expressing neural crest cells led to a number of cranial bone defects, including cleft palate and subsequent perinatal death in mice[95]; menin inactivation in mature osteoblasts using osteocalcin-cre mice resulted

TABLE 38.5 Indications for Genetic Testing in MEN1

Suggested indications for germline MEN1 genetic testing
In an index case
• Meeting clinical criteria for MEN1 (i.e., two or more MEN1-associated tumors or a diagnosis of familial MEN1)
• Suspicious for MEN1 (i.e., multiple parathyroid adenomas <40 years of age, recurrent hyperparathyroidism; gastrinoma, or multiple pancreatic NETs at any age), or "atypical" for MEN1 (i.e., development of two nonclassical MEN1-associated tumors, e.g., parathyroid and adrenal tumor)
A first degree relative of a family member with known *MEN1* mutation
• Asymptomatic first-degree relative
• First-degree relative with familial MEN1 (i.e., one MEN1-associated tumor)[a]

[a]*Testing is indicated in this setting to avoid diagnostic confusion arising from potential phenocopies, which are reported to occur in 5%–10% of MEN1 kindreds.*
Adapted from Newey PJ and Thakker RV. Role of multiple endocrine neoplasia type 1 mutational analysis in clinical practice. Endocr Pract *2011;***17***(Suppl. 3):8–17, with permission.*

in reduced BMD, trabecular bone volume and cortical bone thickness[96]; and transgenic overexpression of osteoblast-specific menin facilitated a gain of bone mass with increased osteoblast number thereby further substantiating a key role for menin in bone development.[96]

2.14 MEN1 Mutational Analysis in Clinical Practice

MEN1 mutational analysis, as recommended by current guidelines, is helpful in clinical practice[2] in several ways that include: (1) confirmation of the clinical diagnosis; (2) identification of family members who harbor the *MEN1* mutation and require screening for tumor detection and early treatment; and (3) identification of family members who do not harbor the familial germline *MEN1* mutation and can therefore be reassured.[2,12] Current guidelines also recommend that *MEN1* mutational analysis should be undertaken in asymptomatic first-degree relatives of a patient with MEN1, at the earliest opportunity and, where possible, in the 1st decade of life as tumors may develop in early childhood (Table 38.5).[2,12] While, clinically relevant disease is uncommon in those younger than 5 years, primary hyperparathyroidism, insulinoma, and adrenal cortical tumors have been reported.[6] Thereafter, recent studies indicate a penetrance of ~15% by 10 years and >50% by 20 years, indicating the potential value of early genetic diagnosis.[6] Although it is not possible to predict either the timing or spectrum of tumors in MEN1, specific manifestations may occur more frequently in individual kindreds. For example, thymic carcinoids may cluster in families with incomplete penetrance, while pituitary and adrenal disease have also been reported to display such intrafamilial correlations.[97] In addition, recent studies have suggested that *MEN1* mutations affecting the JunD interaction domain are associated with an increased risk of death,[98] while those affecting the CHES1-interaction domain are associated with aggressive behavior of P-NETs.[99] The current guidelines recommend that individuals at high risk for MEN1 (i.e., mutant gene carriers) undergo periodic clinical, biochemical, and radiological screening (Table 38.6; Fig. 38.3) although the frequency and scope of investigation is debated.[2]

3 MULTIPLE ENDOCRINE NEOPLASIA TYPES 2 AND 3 (MEN2 AND MEN3)

Multiple endocrine neoplasia type 2 (MEN2) has a reported of incidence 1/80,000–200,000 live births and is characterized by the occurrence of medullary thyroid

TABLE 38.6 Summary of Biochemical and Radiological Screening Guidelines in Individuals at High Risk of Developing MEN1

Tumors	Age to begin (years)	Biochemical test (annually)	Imaging test (time interval)
Parathyroid	8	Calcium, PTH	None
Gastrinoma	20	Gastrin (± gastric acid output, gastric pH)	None
Insulinoma	5	Fasting glucose, insulin	None
Other enteropancreatic	<10	Chromogranin-A; glucagon; pancreatic polypeptide, vasoactive intestinal peptide	MRI, CT, EUS (annually)
Anterior pituitary	5	PRL, IGF-1	MRI (every 3 years)
Adrenal	<10	None, unless signs or symptoms of functioning tumor and/or >1 cm tumor identified on imaging	MRI or CT (annually with pancreatic imaging)
Foregut carcinoid	15	None	CT or MRI (every 1–2 years)

CT, Computer tomography; EUS, endoscopic ultrasound; IGF-1, insulin growth factor-1; PTH, parathyroid hormone; PRL, prolactin; MRI, magnetic resonance imaging.
Adapted from Thakker et al. Clinical practice guidelines for multiple endocrine neoplasia type 1 (MEN1). J Clin Endocr Metabol *2012;***97***:2990–3011, with permission.*

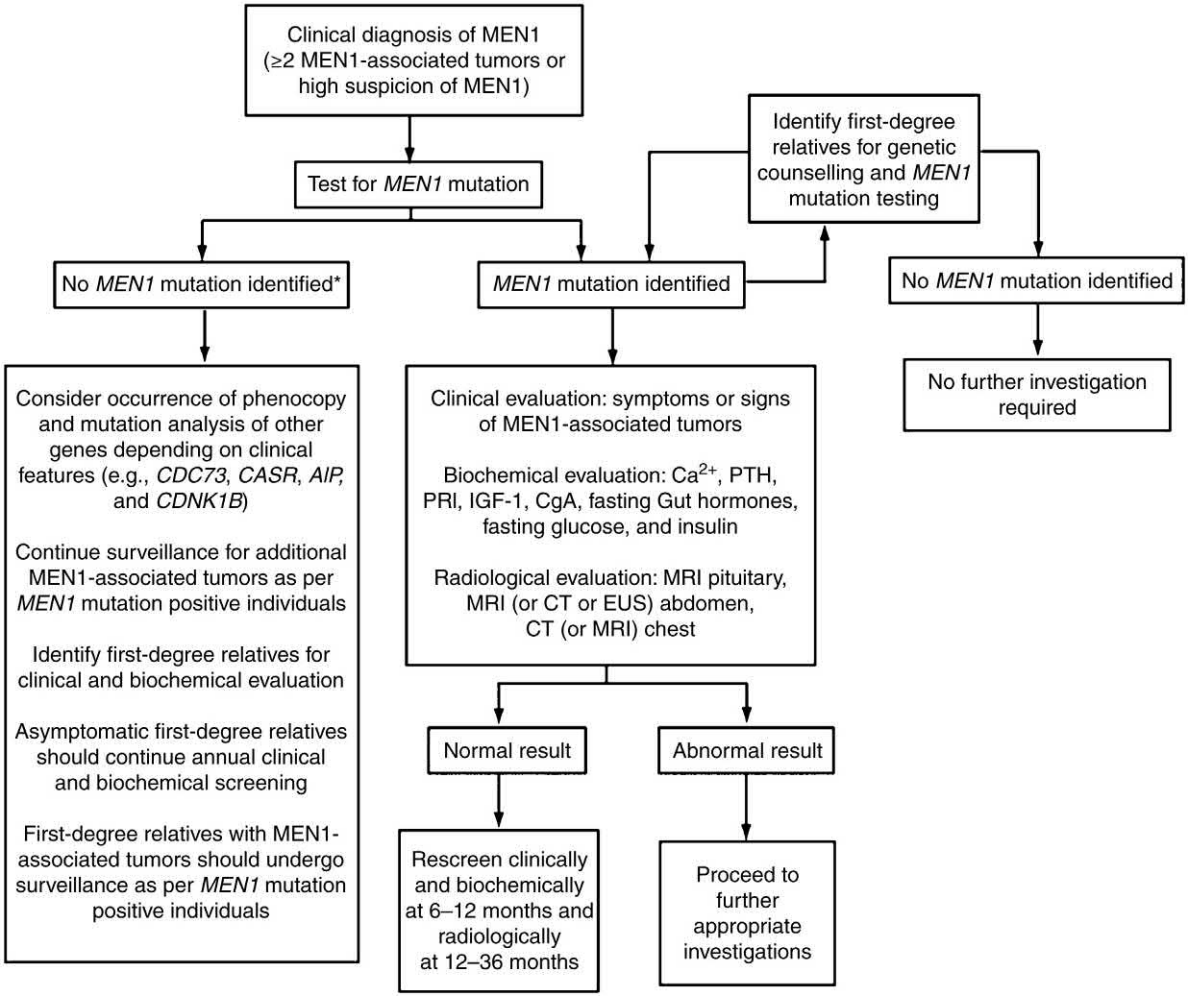


FIGURE 38.3 An approach to screening in MEN1. Index cases, or individuals in whom there is a high suspicion of clinical MEN1 (e.g., multigland parathyroid disease, parathyroid, and adrenal tumor), should be offered genetic counseling and *MEN1* mutation testing. Mutation testing should also be offered to those with familial MEN1 (i.e., individual with one MEN1-associated tumor and a first-degree relative with a known *MEN1* mutation). The identification of a germline *MEN1* mutation should prompt entry into a periodic clinical, biochemical, and radiological screening program. At the same time, first-degree relatives should be identified and offered genetic counseling and *MEN1* mutation testing. Individuals who have inherited the *MEN1* mutation should enter periodic screening, even if asymptomatic. First-degree relatives who have not inherited the *MEN1* mutation require no further follow up and may be alleviated of the anxiety associated with the development of MEN1-associated tumors. For index cases, in whom a *MEN1* mutation, which includes testing for partial or whole gene deletions (asterisked), is not identified, additional genetic testing may be required depending on the specific clinical features. This may include examination for mutations in genes associated with familial parathyorid syndromes, including *CDC73* associated with the hyperparathyoridism-jaw tumor syndrome (HPT-JT) and the calcium sensing receptor (*CASR*) associated with familial benign hypercalciuric hypercalcemia (FBHH); or cyclin-dependent kinase 1B (*CDKN1B*) and aryl hydrocarbon receptor interacting protein (*AIP*), which are rarely identified in those with clinical MEN1. Up to 10% of kindreds with clinical MEN1 may harbor phenocopies emphasising the importance of accurate genetic evaluation. For MEN1 kindreds in whom no *MEN1* mutation is identified, a pragmatic approach is to offer clinical, biochemical, and radiolical screening to those with clinical manifestations of disease and to offer annual clinical and biochemical screening to asymptomatic first-degree relatives. *Ca^{2+}*, Calcium; *CgA*, chromogranin A; *CT*, computer tomography; *EUS*, endoscopic ultrasound; *IGF-1*, insulin-growth-factor-1; *MRI*, magnetic resonance imaging; *PRL*, prolactin; *PTH*, parathyroid hormone. *Source: Reproduced from Thakker RV, Newey PJ, Walls GV, et al. Clinical practice guidelines for multiple endocrine neoplasia type 1 (MEN1).* J Clin Endocrinol Metab *2012;***97***(9):2990–3011, with permission.*

carcinoma (MTC) in association with pheochromocytoma and parathyroid tumors (Table 38.1).[100,101] Historically, three clinical syndromes have been recognized and are referred as MEN2A, MEN2B, and MTC-only, although recent guidelines suggest the MTC-only category should be considered a variant of MEN2A.[102] Thus, current guidelines identify two syndromes: MEN2A, now referred to as MEN2, and MEN2B, now referred to as MEN3.[102] In classical MEN2A, which is the most common clinical variant accounting for 90%–95% of cases, the development of MTC is associated with pheochromocytoma and parathyroid tumors. Three additional MEN2A variants are reported; MEN2A with cutaneous lichen sclerosis; MEN2A with Hirschsprung's disease;

and the MTC-only variant. MEN2B occurs much less frequently than MEN2A, representing ~5%–10% of cases. MEN2B often presents in infancy and is associated with an aggressive disease course.[100,101] In contrast to MEN2A, parathyroid involvement is rare in MEN2B, which is characterized by the occurrence of MTC and pheochromocytoma in association with a marfanoid habitus, mucosal neuromas, medullated corneal fibers, and intestinal autonomic ganglion dysfunction leading to megacolon.[100,101]

Each of the clinical variants of MEN2 is inherited as an autosomal dominant disorder and is due to germline mutations in the *rearranged during transfection* (*RET*) protooncogene.[101,103] However, de novo RET mutations are observed in ~5%–10% and ~50%–75% of cases of MEN2A and MEN2B, respectively. A strong genotype–phenotype correlation is observed in MEN2A, such that both the clinical expression of disease and aggressiveness of MTC is determined by the codon-specific *RET* mutation.[101] While MEN2A is increasingly diagnosed genetically and facilitates the careful monitoring and treatment of asymptomatic individuals, the diagnosis of MEN2B is frequently delayed as the majority of cases occur sporadically (i.e., due to de novo *RET* mutation).

3.1 Medullary Thyroid Cancer

MTC is a malignant neoplasm of the thyroid parafollicular C cells and is most highly concentrated in the middle and upper regions.[101–103] As a result, MEN2-associated MTC is typically observed in these locations and is typically multifocal and bilateral. MTC is reported to evolve through a multistep process starting with C cell hyperplasia, followed by noninvasive microscopic MTC, subsequently progressing to invasive carcinoma with lymph node and distant metastatic spread.[101] Parafollicular C cells secrete several peptide and biogenic amine hormones, including calcitonin, which provides a valuable diagnostic and surveillance marker for MTC.[101,104] Surgery is the mainstay of treatment for MTC and early diagnosis, prior to the development of metastatic disease is required for curative approaches.

3.1.1 Clinical Findings

MTC is highly penetrant in MEN2, such that 70%–100% of affected individuals will develop disease by 70 years of age. MTC is typically the first manifestation of MEN2 and frequently presents in childhood.[101] Adult presentation may occur but is typically associated with less aggressive and/or penetrant *RET* mutations. MTC remains the major determinant of premature mortality in MEN2 patients due to its high malignant potential. However, the identification of *RET* as the causative gene and the ability to undertake genetic testing has resulted in a shift in clinical presentation from those presenting with a neck mass and advanced disease to asymptomatic individuals identified as "at-risk." For an index case in which there is no apparent family history, the presentation is typically with a palpable neck mass. Additional symptoms include diarrhea reflecting high circulating concentrations of calcitonin or other tumor-secreted hormones. Flushing may also occur with features similar to the carcinoid syndrome, while ectopic production of ACTH may given rise to Cushing's syndrome.[44] Lymph node and distant metastases are often present at the time of diagnosis in those presenting with a neck mass.[102,105] The cervical and mediastinal nodes are the most common sites of local metastases while distant spread typically involves bone, liver, lung, or brain.[102,106]

The MTC diagnosis is dependent on the demonstration of high basal calcitonin levels together with supporting radiological and/or cytological evidence.[102] For individuals presenting with a solitary thyroid nodule, fine-needle aspiration is the initial investigation of choice. Once MTC is suspected, measurement of plasma basal calcitonin levels is indicated, although a variety of situations may give rise to false positive plasma calcitonin elevations, including chronic renal failure, parathyroid disease, thyroiditis, lung and prostate cancers, while false-negative results may rarely occur due to a "Hook effect" in which very high levels of serum calcitonin saturate the binding capacity of the antibody in the immunoassay. In addition, occasional patients with advanced MTC may have relatively low levels or calcitonin due to marked dedifferentiation of the cancer.[102] Of note, calcitonin levels may be elevated in healthy infants. In addition to calcitonin, measurement of carcinoembryonic antigen may be useful for monitoring disease progression.[104,107] Preoperative staging with neck ultrasound is mandatory while those with evidence of extensive neck disease or very high calcitonin levels require cross-sectional imaging and bone scintography to detect distant spread.[102]

3.1.2 Treatment

In individuals with clinically evident MTC the extent of surgery depends on the degree of local and distant involvement, although consensus on the extent of surgery remains controversial. However, recent guidelines provide recommendations based on the available evidence.[102] Curative surgery typically involves total thyroidectomy with central compartment dissection with or without unilateral or bilateral lateral neck dissection, while in those with more advanced disease (i.e., extensive regional or metastatic disease) less aggressive surgery may be appropriate as cure will not be achieved. Prognostic information is provided by TNM staging, the number of lymph node metastases, and postoperative calcitonin level, while the postoperative calcitonin doubling time, estimated by calcitonin

measurement every 6 months, provides additional useful information.[102,104,105]

For those with advanced disease a number of potential treatment modalities are available, including further surgical exploration and external beam radiotherapy. Cytotoxic chemotherapy is associated with low response rates and is not typically recommended first line therapy. Radionuclide therapies have been employed in small studies with apparent improved survival (e.g., [^{90}Y-DOTA]-TOC). Recently, the emergence of targeted therapies employing TKIs, has provided some optimism in the treatment of advanced MTC. Both vandetanib (targeting RET, EGFR, and VEGFR kinases) and cabozantinib (targeting RET, c-Met, and VEGFR) have each been evaluated in prospective randomized phase III clinical trials of patients with advanced MTC and are associated with objective tumor response and improvements in progression free survival, although over time, the emergence of resistance occurs with subsequent disease progression.[102,108,109]

3.1.3 Prophylactic Surgery

The ability to identify individuals at high-risk of developing hereditary MTC through germline *RET* mutation testing provides the opportunity to undertake preventative or curative surgery in asymptomatic individuals. However, the timing of surgery remains controversial. The goal of "prophylactic" thyroidectomy is not necessarily to remove the thyroid before any abnormality develops (e.g., C cell hyperplasia) but to do so before there is significant risk of metastatic disease. The age of onset and aggressiveness of MTC in MEN2 is dependent both on the specific *RET* genotype, as well as yet unidentified factors. Guidance on the timing of prophylactic thyroidectomy is governed according to risk stratification of *RET* mutations, and recent guidelines suggest three levels of risk classed as "highest," "high," and "moderate" (Tables 38.7 and 38.8).[102] Prophylactic thyroidectomy with life-long thyroxine replacement, has dramatically improved outcomes in patients with MEN2 and MEN3, such that ~90% of young patients with *RET* mutations who had a prophylactic thyroidectomy have been reported to have no evidence of persistent or recurrent MTC at 7 years after surgery.

3.2 MEN2- and MEN3-Associated MTC and Relevance to Skeletal Disease

There are no known direct skeletal phenotypes associated with MEN2 or MEN3. However, MEN2 and MEN3 patients may be at risk of bone disease related to the

TABLE 38.7 Clinical Relationships and MTC risk Level Associated With Common *RET* Mutations in MEN2A and MEN2B (MEN3)

Exon	Affected codon/ mutation	ATA MTC risk level	Penetrance of pheochromocytoma (%)	Penetrance of primary HPT (%)	Additional reported associations
8	G533C	Moderate	c.10	c.10	—
10	C609F/G/R/S/Y	Moderate	c.10–20	c.10	HD
10	C611F/G/S/Y/W	Moderate	c.10–20	c.10	HD
10	C618F/R/S	Moderate	c.10–20	c.10	HD
10	C620F/R/S	Moderate	c.10–20	c.10	HD
11	D631Y	Moderate	c.50	—	—
11	C634 F/G/R/S/W/Y	High	c.50	c.20–30	CLA
11	K666E	Moderate	c.10	—	—
13	K768D	Moderate	—	—	—
13	L790F	Moderate	c.10	—	—
14	V804L	Moderate	c.10	c.10	—
14	V804M	Moderate	c.10	c.10	—
15	A883F	High	c.50	—	—
15	S891A	Moderate	c.10	c.10	—
16	R912P	Moderate	—	—	—
16	M918T	Highest	c.50	—	MEN2B clinical features

CLA, Cutaneous lichen sclerosis; HD, Hirschsprung's Disease; MTC, medullary thyroid carcinoma; —, not typically observed/associated.
Adapted from Wells et al. Revised American Thyroid Association guidelines for the management of medullary thyroid carcinoma. Thyroid *2015;***25***:567–610.*

TABLE 38.8 Recommendations for Screening and Surgery in MEN2 and MEN2B (MEN3)

ATA risk category[a]	Relevant *RET* mutations	Recommended age (years) for screening test/intervention				
		***RET* mutational analysis**	**First serum calcitonin and neck USS**	**Prophylactic thyroidectomy**	**Screening for PCC[b]**	**Screening for primary HPT**
Moderate	Validated pathogenic mutations excluding those in high and highest category[c]	<3–5	5	>5[d]	16	16
High	C634F/G/R/S/W/Y A883F	<3	3	5 or earlier[e]	11	11
Highest	M918T[f]	ASAP and by <1	ASAP and by <0.5–1	ASAP and by <1	11	—

ASAP, As soon as possible; ATA, American Thyroid Association; HPT, hyperparathyroidism; PCC, pheochromocytoma; USS, ultrasound; —, not required as not part of MEN2B (MEN3).

[a] *ATA risk category as defined in the "Revised American Thyroid Association guidelines for the management of medullary thyroid carcinoma."* Thyroid *2015;***25***:567–610.*

[b] *Individuals with MTC must have pheochromocytoma excluded prior to a surgical intervention, and should be excluded in all at risk individuals who are planning pregnancy or are pregnant.*

[c] RET *mutations reported at ClinVar, ARUP database (arup.utah.edu/database/MEN2/MEN2_welcome.php).*

[d] *Timing of surgery to be based on elevation of serum calcitonin and/or the joint discussion of the pediatrician, surgeon, and parent/family. For example, later surgery may be appropriate if serum calcitonin and neck ultrasound are normal.*

[e] *Earlier than 5 years based on elevation of serum calcitonin. The surgeon and pediatrician in consultation with the child's parent should decide the optimal timing of surgery.*

[f] RET *mutation associated with MEN2B.*

Adapted and updated from The American Thyroid Association Guidelines Task Force, Kloos RT, Eng C, Evans DB, Francis GL, Gagel RF, et al. Medullary thyroid cancer: management guidelines of the American Thyroid Association. Thyroid *2009;***6***:565–612 and Wells et al. Revised American Thyroid Association guidelines for the management of medullary thyroid carcinoma.* Thyroid *2015;***25***:567–610.*

associated tumors and their treatment and this is briefly discussed.

3.2.1 Skeletal Involvement in Metastatic MTC

Skeletal involvement in MTC is common in those with advanced disease. Early reports indicated a frequency of bone involvement in ~30% of those with advanced disease.[106] More recently, a large institutional study reported bone involvement in ~5% of a cohort of 715 individuals with MTC, and in ~30% of those with advanced disease.[105] Isolated bone involvement may occur in those with distant metastases although more commonly occurs in those with liver and/or lung spread.[105] While the extent of nodal involvement is reported to act as a good predictor of lung and liver metastases, it did not predict bone involvement.[105] Higher rates of skeletal involvement (i.e., 70% of individuals advanced disease) have been observed when sensitive imaging modalities, including axial MRI and postradioiodine scintography have been employed.[106] The most frequently affected skeletal sites include the spine and pelvis and multimodal imaging techniques may be required to detect disease and these include MRI of the spine and pelvis, bone scintography, and FDG-PET-CT.[102,106] Additional nuclear medicine-based modalities, including F-DOPA-CT-PET may also be of value where available. Treatment of skeletal metastases includes vertebroplasty, local excision or ablation, and external beam radiotherapy.[102,110] Bone pain and/or risk of further bone involvement may be reduced by administration of bisphosphonates or the receptor activator of nuclear factor kappa-B ligand (RANKL) inhibitor denosumab.[111]

3.2.2 Role of Calcitonin on Bone Metabolism

MTC is characterized by increased levels of calcitonin, a 32 amino acid polypeptide, which under physiological conditions is secreted by the parafollicular C-cells of the thyroid gland.[112,113] Studies, primarily from animal models, indicate that calcitonin has important effects on calcium homeostasis and bone health, yet despite extensive investigation, its role in human physiology remains elusive, not least reflecting a lack of clinical phenotypes associated with hormone excess or deficiency. For example, neither individuals with MTC and hypercalcitoninemia, nor those with apparent calcitonin deficiency following total thyroidectomy, demonstrate alterations in BMD compared to control individuals.[114–116] Thus, the significance of alterations in calcitonin levels on human bone health appears at most modest. A brief overview of calcitonin-mediated skeletal effects established from animal models is therefore provided.

Calcitonin is derived from the alternate splicing of the *CALCA* gene, which also encodes the neuropeptide α-calcitonin gene-related peptide (α-CGRP).[112,113] Calcitonin is a hypocalcemic hormone, and is secreted in response to hypercalcemia. Indeed, pharmacological doses of salmon calcitonin have been used for the treatment of hypercalcemia and osteoporosis in patients.[112,113] The physiological relevance of calcitonin's hypocalcemic activity remains ill defined, as are its effects on bone metabolism demonstrated in calcitonin knockout mice. For example, $Calca^{-/-}$ mice (resulting in loss of both calcitonin and α-CCGRP) have normal calcium and bone-related biochemistry, although they demonstrate an enhanced hypercalcemic response to PTH presumed,

which is presumed secondary to prolonged bone resorption.[116–118] Surprisingly, *Calca*$^{-/-}$ mice also reveal increased bone formation at 1–3 months of age.[117] However, in contrast to this anabolic effect on bone formation which is sustained throughout life, aged *Calca*$^{-/-}$ demonstrate marked cortical porosity and reduction in trabecular thickness secondary to increased bone resorption.[119] Thus, these studies highlight apparent paradoxical roles for calcitonin in the regulation of bone formation and resorption.[116,117,119] Furthermore, conventional and conditional calcitonin receptor knockout mice (*Calcr*$^{-/-}$) support these observations demonstrating increased bone formation, a response apparently mediated by increased expression of sphingosine 1-phosphate.[120] Such studies indicate that targeting the calcitonin pathway may hold promise for the development of anabolic bone therapies.[120] Murine models also indicate that calcitonin may play a key role in protecting the maternal skeleton during lactation as *Calcr*$^{-/-}$ mice demonstrate significantly greater reductions in BMD during lactation than wild type mice.[121] The significance of each of these findings remains to be determined in humans.

3.2.3 *Hypoparathyroidism Following Thyroid Surgery*

Both therapeutic and prophylactic thyroid surgery place the adjacent parathyroid glands at functional risk, either through disruption to their blood supply or necessary/inadvertent removal. As a consequence patients with MEN2 or MEN3 undergoing thyroidectomy are at risk of developing postoperative hypoparathyroidism.[102] In the preventative setting, a recent study evaluating the practice of preserving the parathyroid glands in situ where possible, demonstrated a rate of postoperative permanent hypoparathyroidism of 1%,[122] indicating that when possible, viable parathyroid tissue should be preserved in situ. The treatment of iatrogenic hypoparathyroidism typically requires vitamin D analogues and lifelong monitoring. The consequences of hypoparathyroidism on bone are discussed elsewhere (Chapter 34).

3.2.4 *Ectopic Cushing's/Osteoporosis*

MTC may be associated with ectopic hormone production, which includes that of CRH and ACTH that give rise to ectopic Cushing's syndrome.[44] MTC represents 1%–3% of cases of ectopic Cushing's syndrome[102] and causes glucocorticoid-induced osteoporosis.

3.2.5 *Use of TKIs and Effects on Bone*

The TKIs vandetanib and cabozantinib have been approved for the treatment of advanced and metastatic MTC, respectively.[102] As a class of drugs TKIs have been reported to have variable effects on bone mineral metabolism.[123] Vandetanib is a small molecule multikinase inhibitor with activity against the RET protooncogene. Increased rates of hypocalcemia were observed in individuals treated with vandetanib compared with placebo in the context of metastatic MTC.[108] However, in another study, vandetanib treatment was not associated with changes in plasma calcium levels but resulted in increased requirement for calcium and vitamin D replacement in individuals with locally advanced MTC,[123,124] indicating that patients with established hypoparathyroidism, may be more vulnerable to hypocalcemia as they may have an inability to increase their endogenous PTH and 1,25-OH-vitamin D levels.[123] Cabozantinib is a nonselective multikinase inhibitor with activity against RET, VEGFR2, and MET. In the context of prostate cancer, phase II clinical trials reported marked effects on skeletal metastases in terms of reduction in bone turnover markers, tumor response on imaging, and reduction in bone pain.[125] Studies in mice receiving cabozantinib treatment revealed effects on the bone microenvironment with transient reductions and increases in the numbers of osteoclasts and osteoblasts respectively; and alterations in trabecular bone structure and the epiphyseal growth plate.[126] The mechanisms responsible for such effects remain unknown but they may include targeting of the hepatocyte growth factor/MET pathway.[123]

3.3 Pheochromocytoma

3.3.1 *Clinical Findings*

Pheochromocytoma develops in ~50% of patients with MEN2, with the highest occurrence being in those with *RET* codon 634 and Met918Thy mutations (Table 38.7).[102,127–129] Typically, pheochromocytoma presents in the 3rd or 4th decade of life in MEN2A and is often diagnosed concurrently with MTC, although may occur earlier in those with "high" and "highest" risk *RET* mutations (Tables 38.7 and 38.8).[127] Pheochromocytoma is associated with symptoms and signs of catecholamine secretion, including episodic headache, sweating, palpitations, anxiety, and hypertension although the absence of symptoms does not exclude disease its presence. Biochemical testing includes measurement of plasma and/or urinary free fractionated metanephrines. MEN2-associated pheochromocytomas are typically adrenergic and are reported to secrete disproportionate amounts of epinephrine.[128,129] Diagnostic imaging is as for sporadic disease with the use of cross-sectional CT or MRI scanning, and functional imaging (e.g., metaiodobenzylguanidine scanning). In contrast to sporadic pheochromocytoma, bilateral disease is often observed in MEN2A patients and particularly in those with high-risk *RET* mutations.[129] The rate of extraadrenal disease is low with the majority of tumors arising from the adrenal medulla.

3.3.2 *Treatment and Screening*

The treatment of pheochromocytoma does not differ from that of sporadic disease. Surgery is the treatment of

choice with the majority of individuals harboring benign disease. Pre- and perioperative alpha- and betablockade are required and adrenalectomy or adrenal-sparing approaches may be used. Although the risk of bilateral disease is high, bilateral adrenalectomy is not recommended for individuals with unilateral disease.[102] Patients requiring removal of both adrenal glands (i.e., due to synchronous or asynchronous bilateral pheochromocytoma) require life-long glucocorticoid and mineralocorticoid replacement therapy. Screening for pheochromocytoma in MEN2A is recommended (Table 38.8) and should include measurement of plasma or urinary free metanephrines. It is important to exclude pheochromocytoma in all patients with MEN2 prior to general anesthesia and in women of child-bearing age prior to pregnancy.

The potential for skeletal impacts from pheochromocytoma in MEN2 are minimal but include the theoretical involvement from skeletal metastases (albeit extremely rarely) due to malignant pheochromocytoma, and indirect effects via occasional ectopic ACTH secretion.

3.4 Primary Hyperparathyroidism

3.4.1 Clinical Features and Treatment

The penetrance of primary hyperparathyroidism in MEN2A is related to genotype. Overall ~10%–30% of individuals with MEN2A develop primary hyperparathyroidism, with the highest prevalence in those with *RET* codon 634 mutations (Table 38.7).[128,130,131] Frequently the disease is mild and patients are often asymptomatic. Furthermore, patients with enlarged parathyroid glands found at surgery for MTC may be normocalcemic. Screening for PHPT is recommended in patients with MEN2A (Table 38.8).[102] Multigland involvement is typically observed and current recommendations favor surgical removal of only visibly enlarged glands.[102,132] For those with four gland involvement subtotal parathyroidectomy or total parathyroidectomy with autograft approaches have been advocated.

Hyperparathyroidism in MEN2 may result in skeletal manifestations as found in sporadic disease. However, the specific features of the bone abnormalities associated with MEN2A-associated primary hyperparathyroidism have not been established. Initially, the observed hyperparathyroidism was hypothesized to be secondary to high levels of calcitonin but this has not been borne out be subsequent studies. The effects of primary hyperparathyroidism on bone are likely to be similar to those discussed earlier.

3.5 Other Manifestations: Hirschsprung's Disease and Cutaneous Lichen Sclerosis

About 7% of patients with MEN2A manifest features of Hirschsprung's disease, and typically present shortly after birth with the inability to pass stool and the development of megacolon. The cooccurrence of MEN2A with Hirschsprung's disease appears paradoxical as loss-of-function *RET* mutations are observed in sporadic cases of Hirschsprung's disease in contrast to the gain-of-function *RET* mutations observed in MEN2A.[133] Cutaneous lichen sclerosis is rarely observed in MEN2A and almost exclusively occurs in a proportion of individuals with codon 634 mutations.[134] Cutaneous lichen sclerosis typically presents with intense pruritus and a rash in the interscapular region in the T2-6 dermatome region. The lesions are improved with sunlight and may worsen at times of stress.

3.6 Genetics

Following the identification of the *RET* protooncogene, more than 100 mutations have been reported in association with hereditary MTC and MEN2.[100,128,135] The majority of mutations involve nonsynonymous amino acid substitutions in key residues, although duplications, insertions, and deletions of the *RET* gene have also been identified.[128,135] The majority of MEN2-associated *RET* mutations affect a relatively small number of codons. The codon 634 cysteine residue is most frequently disrupted, while mutations in other cysteine residues at codons 609, 611, 618, or 620, and the valine 804 residue, collectively contribute a significant proportion of the remaining MEN2A cases.[100,135,136] In contrast, ~95% of individuals with MEN2B harbor the methionine to threonine mutation affecting codon 918 (Met918Thy), with a much smaller number having mutation of codon 883 (Ala883Phe), although a recent study indicates that individuals with the Ala883Phe mutation have a less aggressive disease course.[137,138] A recent study of ~500 MEN2 families from Germany, Italy, and France demonstrated that ~34% of kindreds harbor codon 634 mutations, with ~17% having codon 804, ~10% codon 918, and 7.6% codon 790 mutations.[100] These studies also estimated the incidence of *RET* mutations in the general European population to be ~1:100,000.[100]

Genetic testing for *RET* mutations has been widely implemented. When a family with a *RET* mutation is known, genetic testing of the specific sequence variant should be performed in all "at-risk" individuals (i.e., first-degree relatives). For index cases with apparent MEN2 and unknown *RET* status, either a prioritized sequencing approach incorporating recurrently affected exons or sequencing of the entire coding region should be undertaken. *RET* genetic testing is also indicated in all individuals with apparently sporadic MTC,[102,139] as well as in first-degree relatives of patients with hereditary MTC, parents whose children have MEN2B, and patients with cutaneous lichen sclerosis. Finally, it has been reported that several apparently benign polymorphic *RET* alleles may act as genetic modifiers in those with established pathogenic *RET* variants, although further clarification of this is required.

3.7 Functions of the *RET* Oncogene and Relevance to Skeletal Biology

The RET protooncogene encodes a single-pass transmembrane receptor of the tyrosine kinase family. The *RET* gene is located on chromosome 10q11.2 and comprises 21 exons.[103,140] The protein consists of three functional domains; an extracellular domain containing four cadherin-like repeats and a cysteine rich region, a hydrophobic transmembrane domain, and a cytoplasmic tyrosine kinase domain.[103,140,141] The protein is expressed in cells derived from the neural crest, including neuroendocrine cells (e.g., thyroid C-cells and adrenal medullary cells), neuronal cells including both sympathetic and parasympathetic ganglion cells, and urogenital tract cells.[101,103,142] Ligands for the receptor are members of the glial cell-line derived neurotropic factor-family, which require glial cell-line derived neurotropic factor-family α coreceptors.[141] Ligand binding results in receptor dimerization, intracellular tyrosine autophosphorylation, and subsequent activation of downstream signaling pathways.[103] The cysteine rich region in the distal extracellular domain is highly conserved and involved in receptor dimerization and it is these cysteine residues that are most frequently disrupted in MEN2. The phosphorylation of key tyrosine residues in the intracellular domain facilitate activation of downstream signaling cascades.[101,103,140]

RET is reported to play a critical early role in neural crest development and is subsequently required for normal development of the enteric nervous system, gut-associated lymphoid tissue, parasympathetic nervous system, kidney, and spermatogenesis.[101,103] Loss-of-function *RET* mutations are observed in Hirschsprung's disease in which a defect in migration and development of enteric neurons is observed, while the gain-of-function mutations observed in MEN2A and MEN2B result in enhanced receptor activation and signaling. However, the absence of *RET* expression in bone lineages, and the lack of a defined bone phenotype in *Ret* transgenic or knockout mice indicate that *RET* is unlikely to have a major role in bone biology.[142–144]

4 MULTIPLE ENDOCRINE NEOPLASIA TYPE 4 (MEN4)

Approximately 5%–10% of patients with a clinical diagnosis of MEN1 do not have a mutation of the *MEN1* gene and of these ~3% will have a mutation in the *CDKN1B* gene. Such individuals are referred to as having MEN4.[1,145]

4.1 Clinical Findings and Treatment

To date only a small number of individuals and/or kindreds have been reported with MEN4 and hence phenotypic information is limited.[1] Each of the affected individuals had primary hyperparathyroidism, as well as somatotrophinomas, corticotrophinomas, and nonfunctioning pituitary adenomas, adrenal cortical tumors, pancreatic NETs, thyroid, and uterine neoplasms.[1,146] In the absence of detailed phenotypic data, the recommended investigation and treatment is as for MEN1-assocaited tumors.

4.2 Genetics and Screening

MEN4 is inherited as an autosomal dominant disorder due to heterozygous mutation of the *CDNKIB* gene located on chromosome 12p13. *CDKN1B* encodes the 196 amino acid cyclin-dependent kinase inhibitor ($p27^{kip1}$). To date the small number of *CDKN1B* mutations (~10 reported in the literature to date) are reported to result in reduced levels of $p27^{Kip1}$ or altered protein function consistent with a tumor suppressor role.[146] Germline *CDNK1B* mutations have also been reported in patients with apparent sporadic (i.e., nonfamilial) primary hyperparathyroidism although further studies are required to confirm whether such individuals have isolated parathyroid disease or develop additional MEN-associated tumors. Currently, undertaking genetic analysis for *CDKN1B* mutations is indicated in individuals or kindreds with clinical evidence of MEN1 but no *MEN1* mutations, in individuals with an atypical MEN1 presentation negative for a *MEN1* mutation, or in first-degree relatives of those with a known *CDKN1B* mutation. Surveillance for tumor development should be as for MEN1.

5 MULTIPLE ENDOCRINE AND OTHER ORGAN NEOPLASIAS (MEONS) SYNDROMES

MEONs syndromes, include the HPT-JT, NF1, VHL, CNC, CWD, and MAS. These syndromes will be briefly reviewed together with their skeletal features.

6 HYPERPARATHYROIDISM-JAW TUMOR SYNDROME

6.1 Clinical Findings and Treatment

HPT-JT syndrome is an autosomal dominant disorder characterized by the development of parathyroid tumors in association with ossifying fibromas of the maxilla and/or mandible,[147,148] and due to mutations of the cell cycle division 73 (*CDC73*) gene. In addition, some patients may also develop Wilms' tumors, renal cysts, renal hematomas, renal cortical adenomas, papillary renal cell carcinomas, pancreatic adenocarcinomas, uterine tumors, testicular mixed germ cell tumors, and Hurthle cell thyroid adenomas.[147–149] Approximately 85% of HPT-JT

patients will have a single parathyroid adenoma, but the remaining ~15% of HPT-JT patients will have a parathyroid carcinoma; some patients may have multigland parathyroid disease. Ossifying fibromas of the maxilla and mandible occur in ~25%–50% of HPT-JT patients. The investigation and treatment for the HPT-JT associated tumors is similar to that in non-HPT-JT patients although early parathyroidectomy is advisable because of the increased occurrence of parathyroid carcinoma.[147]

6.2 HPT-JT and Relevance to Skeletal Disease

The ossifying fibromas observed in patients with HPT-JT syndrome typically affect the mandible and/or maxilla and may occur as single or multiple lesions, which may be unilateral or bilateral.[150] Ossifying fibromas represent bone-producing fibrous neoplasms and are distinct from the fibrous dysplasia observed in the MAS due to activating mutations of *GNAS*, despite harboring similar microscopic features.[150,151] The ossifying fibromas typically affect tooth-bearing regions although they may extend to include the mandibular ramus. Radiologically, ossifying fibromas may appear as expansive, lytic lesions, that may have radiolucent and/or radio-opaque properties,[150,151] and histologically they are characterized by a well-defined but unencapsulated hypercellular fibroblast-rich stroma together with calcified bone trabeculae and are indistinguishable from cement-ossifying fibromas.[151] Treatment of the lesions is typically surgical and is primarily undertaking for cosmetic indications. It is noteworthy that both somatic and germline *CDC73* mutations have been reported in apparently sporadic jaw ossifying fibromas.

The consequence of primary hyperparathyroidism on bone health from HPT-JT associated parathyroid tumors has not been specifically investigated but is unlikely to differ significantly from that observed for other primary hyperparathyroid disorders, for example, MEN1 (see earlier).

6.3 Genetics and Screening

HPT-JT is due to mutations of the *CDC73* gene located on chromosome 1q31.2. More than 50 germline heterozygous *CDC73* mutations have been reported in patients with the HPT-JT syndrome, FIHP, and sporadic parathyroid carcinoma.[147,152] Somatic *CDC73* mutations have also been reported in sporadic parathyroid carcinomas, parathyroid adenomas, and ossifying fibromas.[147,152,153] The majority of HPT-JT associated *CDC73* mutations are nonsense or frameshift changes predicted to result in a functional loss of the encoded protein, parafibromin, although large gene deletions have also been reported.[147,154] Genotype–phenotype correlations have not been identified, while apparent nonpenetrance of the disorder is reported in ~30% of mutation carriers. However, parathyroid tumors and ossifying fibromas have been reported in childhood prompting the recommendation for genetic testing at a young age in first-degree relatives of affected individuals.[147] Germline *CDC73* analysis is also indicated in individuals with sporadic parathyroid carcinoma and may also be appropriate in: kindreds with apparent FIHP; patients with sporadic parathyroid disease at a young age; patients with nonfamilial ossifying fibromas of the maxilla or mandible; and in patients with parathyroid disease in association with a clinical manifestation reported in HPT-JT (e.g., renal or uterine tumors). For asymptomatic *CDC73* mutation carriers, biochemical and radiological screening has been advocated (Table 38.9).

6.4 Parafibromin: Function and Relevance to Bone Biology

The protein encoded by *CDC73*, referred to as parafibromin (also known as hCdc73/HRPT protein), is an evolutionary conserved, predominantly nuclear protein, which forms a component of the ubiquitously expressed human RNA polymerase II-associated factor (PAF) complex, involved in transcription processes that include initiation, elongation, and posttranscriptional maturation.[154–156] Indeed, a key function of the PAF complex is undertaking histone modifications associated with active transcription.[157,158] Parafibromin is also

TABLE 38.9 HPT-JT Suggested Guidelines for Screening Patients; Asymptomatic Mutation Carriers; and First- and Second-Degree Relatives in a Family Without Identified Germline *CDC73* Mutations

Tumors[a]	Tests	Frequencies[b]
Parathyroid	Serum Ca, PTH	6–12 months
Ossifying jaw fibroma	Panoramic jaw X-ray with neck shielding[c]	5 years
Renal	Abdominal MRI[c,d]	5 years
Uterine	Ultrasound (transvaginal or transabdominal) and additional imaging ± D&C if indicated[e]	Annual

Ca^{2+}, Calcium; D&C, dilatation and curettage; MRI, magnetic resonance imaging; PTH, parathyroid hormone.

[a]*Screening for most common HPT-JT-associated tumors is considered. Assessment for other reported tumor types may be indicated (e.g., pancreatic, thyroid, and testicular tumors).*

[b]*Frequencies of repeating test after baseline tests performed.*

[c]*X-rays and imaging involving ionising radiation should ideally be avoided to minimise risk of generating subsequent mutations.*

[d]*Ultrasound scan recommended if MRI unavailable.*

[e]*Such selective pelvic imaging should be considered after obtaining a detailed menstrual history.*

*Reproduced from Newey et al. Cell division cycle protein 73 homolog (*CDC73*) mutations in the hyperparathyroidism-jaw tumor syndrome (HPT-JT) and parathyroid tumors.* Hum Mutat *2010;***31***:295–307, with permission.*

a key regulator of Wnt signaling through a direct interaction with β-catenin, as well as influencing Hedgehog signaling.[159,160] More recently it has been reported that parafibromin, located within the cytoplasm, controls the stability of p53 mRNA,[161] and is also a target of SUMOylation, which regulates its cellular localization and function.[162] Little is known regarding the role of parafibromin in bone biology and development, or in formation of ossifying fibromas. Conventional *Cdc73* knockout null mice (*Cdc73*$^{-/-}$) have early embryonic lethality,[156] and the detailed characterization of the skeletal phenotype in conventional heterozygous *Cdc73*$^{+/-}$, or relevant conditional *Cdc73* models, has not been reported. One study has reported that parafibromin may act as a tumor suppressor in osteosarcoma cells through the induction of apoptosis.[163]

7 NEUROFIBROMATOSIS TYPE 1

7.1 Clinical Findings and Treatment

NF1 is an autosomal dominant disorder affecting 1:3000 individuals. The diagnosis of NF-1 is typically a clinical one made by the identification of at least two of the following National Institutes of Health diagnostic criteria: ≥ 6 café-au-lait macules >5mm in diameter in prepubertal individuals or >15mm in diameter postpuberty; ≥2 neurofibromas of any type of a plexiform neurofibroma; freckling in the axillary or inguinal regions; optic glioma; ≥2 Lisch nodules (iris) hamartomas; a distinctive osseous lesion, such as sphenoid dysplasia or tibial pseudoarthrosis; and a first-degree relative with NF-1 as defined by the previous criteria.[164] These criteria may not be evident in infants but the majority of affected individuals will have clinically apparent features by age of 8 years. The presence of a family history makes the diagnosis more straightforward due to the high index of suspicion and requirement for only a single diagnostic criterion to confirm the diagnosis. In addition to the diagnostic clinical features, a wide range of additional manifestations may also occur, including CNS tumors (e.g., brainstem and cerebellar gliomas), malignant peripheral nerve sheath tumors, leukemia and myelodysplastic syndromes, vascular lesions (e.g., stenoses of renal and intracranial arteries, aneurysms, arteriovenous fistula formation), and a spectrum of endocrine abnormalities (e.g., pheochromocytoma, carcinoid tumors, and precocious puberty).[164]

7.1.1 Endocrine Abnormalities

Patients with NF1 have a 1%–5% lifetime risk of developing pheochromocytoma.[164,165] The features of pheochromocytomas in NF1 are similar to those in non-NF1 patients, and the majority of tumors are benign and cured by surgery. Primary carcinoid tumors occur in ~1% of NF1 patients and are often periampullary.[164] Symptoms may relate to biliary dilatation and pancreatitis, or due to the carcinoid syndrome (typically in the presence of hepatic metastases). Gastrinoma, nonfunctioning P-NETs, and insulinoma have also been observed.

Hypothalamic involvement of optic gliomas may disrupt the hypothalamic–pituitary axis and result in growth abnormalities in children, and/or precocious or delayed puberty.[164–166] Precocious puberty is typically associated with an optic glioma extending into the hypothalamus, although this may also rarely occur in the absence such involvement. GH hyper- or hyposecretion may also occur in those with or without optic gliomas, although short stature independent of GH deficiency may result from skeletal abnormalities.[166] The investigation and treatment of the endocrine manifestations in NF1 is similar to that for the sporadic counterparts.

7.2 NF-1 and Relevance to Skeletal Disease

A wide range of musculoskeletal phenotypes are observed in NF1 and provide insights into the consequences of disruption to RAS signaling pathways.[164,167–170] Although a number of additional "RASopathies" have been identified (e.g., Noonan's syndrome and Costello syndrome) with overlapping clinical features, NF1 provides a paradigm for these disorders.[171] The potential range of musculoskeletal phenotypes observed in NF1 includes scoliosis, long bone dysplasia with or without pseudoarthrosis, kyphosis, winged scapula, osteoporosis, bone cysts, bone overgrowth, hand anomalies, impaired fracture healing, and short stature.[168,170,171]

Spinal deformities are the most common skeletal manifestation of NF1 with ~20%–50% of patients having some degree of scoliosis.[168,170,172] NF1-associated scoliosis is traditionally divided into dystrophic and nondystrophic forms according to radiologically-defined features, although this distinction may not accurately reflect the underlying pathophysiological processes. Dystrophic scoliosis is characterized by rib "penciling," vertebral rotation and wedging, spindling of the transverse process, and enlarged intervertebral foramina and typically occurs over a short distance (e.g., 4–6 vertebrae) with a sharp angular curve.[168,170,172] In contrast, nondystrophic scoliosis has a less severe curve and resembles idiopathic adolescent scoliosis. However, nondystrophic scoliosis may become dystrophic and requires careful monitoring. While nondystrophic scoliosis may respond to bracing, dystrophic curvature of sufficient severity (e.g., >20 degree) may require surgical fusion. Kyphosis is also observed in NF1 and may be associated with spinal cord damage as a result of the cord being "stretched" over the sharp angulated vertebrae.[168,172] Atlantoaxial instability and subaxial subluxation may also be observed and

are of particular concern for those undergoing anesthesia.[170] A further spinal problem is dural ectasia, which describes the expansion of the spinal canal and dilatation of the dural sac.[170]

Congenital long bone dysplasia represents a severe skeletal manifestation of NF1 and affects ~5% of patients.[164,168] The tibia is frequently affected and the characteristic unilateral anterolateral bowing of the tibia in a young child may raise the possibility of NF1. Indeed infants may be born with tibial fractures and/or pseudoarthrosis, or these may develop shortly after birth. In those with tibial bowing without fracture, cortical thinning and medullary canal narrowing may be observed. If fractures occur, they typically arise before 3 years of age and rarely heal spontaneously. Multiple surgeries, which involve removal of the pseudoarthrosis and associated fibrous tissue and reconstruction, may be required. Finally osteopenia and osteoporosis are common in patients with NF-1, and children often have reduced BMD.

7.3 Genetics and Screening

NF1 is an autosomal dominant disorder due to heterozygous mutations of the *NF1* gene located on chromosome 17q11.2.[164] However, a significant proportion of *NF1* mutations occur de novo. The *NF1* gene comprises 60 exons and >1400 mutations have been reported scattered throughout the exonic regions.[173] *NF1* mutations are presumed to result in a loss of function as >80% predict premature truncation of the encoded protein. This is supported by the observation of whole gene deletions in association with severe NF1 clinical features. However, additional genotype–phenotype correlations are limited.[173,174] The *NF1* gene product, neurofibromin, is a presumed tumor suppressor and acts as a negative regulator of p21ras by converting the active GTP bound form to its inactive GDP form. Thus, loss-of-function mutation of *NF1* result in activation of Ras signaling cascades [e.g., mitogen activated protein kinase, phosphatidylinositol-3-kinase (PI3K) and mTOR pathways], which in turn may give rise to abnormal cellular phenotypes and aberrant cell proliferation and/or survival.[173] Screening individuals with NF1 for clinical manifestations is recommended and a high index of suspicion for associated lesions should be maintained.

7.4 Neurofibromin Function and Bone

The wide spectrum of bone abnormalities in NF1 patients has prompted investigation into the role of neurofibromin in skeletal tissues. NF1-associated bone lesions, including pseudoarthroses, have been reported to demonstrate loss of heterozygosity and/or somatic *NF1* mutation, indicating that biallelic inactivation with resultant loss of function may contribute to the skeletal phenotype.[175,176] However, heterozygous *Nf1* knockout mice ($Nf^{+/-}$) demonstrate only subtle defects in osteoprogenitor cells and cortical bone, with no overt bone mass or geometry abnormalities.[177] *Nf1*-deficient mouse ($Nf^{-/-}$) embryos die before skeletogenesis commences, and conditional knockout models have therefore been used to investigate neurofibromin function in bone lineages. Thus, use of osteoblast and/or osteoprogenitor specific promoters, conditional *Nf1* depletion has been reported to recapitulate aspects of the human NF1 skeletal phenotype.[169,178–182] For example, depletion of *Nf1* in osteoprogenitor cells, utilizing either *Prx-cre* or *Col2α1-cre* resulted in reduced stature, low bone mass, and bowing of the tibia together with excessive deposition of osteoid, while $Nf1_{col2}{}^{-/-}$ mice also demonstrate progressive scoliosis and kyphosis.[179,181] Aspects of the human skeletal phenotype are also observed in $Nf1^{flox/-}$;*Col1α1-cre* mice, due to activation of the TGF-β 1 signaling pathway, a feature observed in NF1 patients.[182,183] Further support for the involvement of enhanced TGF-β signaling, promoting osteoclast activity, and inhibiting osteoblast differentiation, is provided by the rescue of the murine bone phenotype following administration of a TGF-β kinase inhibitor.[182] However, in contrast to these phenotypes, it is notable that *Nf1* depletion from mature osteoblasts results in a paradoxical increase in bone mass and turnover.[184]

Additional mechanisms contributing to the skeletal abnormalities may include accumulation of pyrophosphate (PP) secondary to chronic activation of the extracellular signal-regulated kinase (ERK) pathway, and an insensitivity to bone morphogenic protein-2 (BMP-2)-induced osteoprogenitor differentiation. Interestingly, the skeletal phenotype in mice with *Nf1*-deficient osteochondroprogenitor cells may be rescued by the administration of asfotase-α enzyme, which facilitates the breakdown of PP, indicating that abnormal mineralization is a key feature of disease.[183] The potential role of ERK activation and BMP insensitivity has been investigated therapeutically. BMP-2 monotherapy does not significantly impact on the skeletal abnormalities in *Nf1* models and in small patient series, but combined BMP-2 treatment with suppression of ERK activation results in bone healing in murine models, thereby indicating that combination therapy with BMP-2 and MEK inhibitors (e.g., Trametinib) may offer a therapeutic avenue for NF1-associated pseudoarthrosis.[185] Activation of the Wnt signaling pathway is also implicated in NF1-associated skeletal disease. Thus, increased β-catenin and Wnt target gene (e.g., AXIN2) expression are observed in pediatric NF1 pseudoarthrosis tissue, a feature shared with a *Nf1* fracture model.[186] Furthermore, the Wnt antagonist Dickkopf-1 can rescue the murine phenotype, as can conditional β-catenin inactivation, thereby indicating that targeting this pathway may hold therapeutic promise.[186]

8 VON HIPPEL–LINDAU DISEASE

8.1 Clinical Findings and Treatments

VHL disease is an autosomal dominant disorder with an incidence of 1:36,000, that is characterized by hemangioblastomas of the retina, and central nervous system (CNS), as well as a spectrum of benign and malignant tumors and/or cysts involving the: kidneys [renal cell carcinomas (RCC), renal cysts], pancreas (cysts, cystadenomas, P-NETs), adrenal glands and sympathetic ganglia (pheochromocytomas and paragangliomas), epididymis (cysts and cystadenomas), and endolymphatic sac tumors.[187,188] Patients typically develop clinically apparent disease between the 2nd and 3rd decade with a mean age of 26 years. Penetrance is high such that >95% of affected individuals will manifest disease by age 65 years. VHL disease is classified into two main categories (Table 38.10). Type 1 includes those with typical manifestations, including hemangioblastoma and RCC, but not pheochromocytoma, while Type 2 describes those with pheochromocytoma and is further subdivided according to the presence of absence of additional manifestations (Table 38.10).[188,189]

The retinal and CNS hemangioblastomas are the most common manifestation of VHL affecting ~70%–80% of patients and are frequently the earliest manifestation of disease.[187] Typically symptoms relate to the site of origin, with CNS hemangioblastomas causing the compression of adjacent structures and/or raised intracranial pressure. The cerebellum is most frequently affected but lesions may occur in the spinal cord and brainstem. Although the majority of hemangioblastomas are benign, they are often highly vascular and morbidity may be associated with tumor enlargement or hemorrhage. The penetrance of RCC is 70% by age 60 years and represents the most frequent malignant tumor in VHL. Metastatic spread is related to tumor size, with lesions <3 cm harboring a good prognosis. Recommended treatment comprises both nephron-sparing surgery and focal ablation.

The endocrine manifestations of VHL primarily consist of pheochromocytomas and P-NETs.[187] The clinical presentation of pheochromocytoma in VHL disease is similar to that in sporadic cases, except that there is a higher frequency of bilateral or multiple tumors, which may involve extra-adrenal sites, although malignancy is rare. VHL should be considered in individuals with apparently sporadic pheochromocytoma.

The most frequent pancreatic lesions in VHL disease are multiple cyst-adenomas, which rarely cause clinical disease.[187] However, nonsecreting P-NETs are observed in ~10% of VHL patients. Affected individuals are frequently asymptomatic and tumors are often detected by radiological screening. P-NETs frequently become malignant, and surgical resection is recommended although the optimal timing of surgery has not been established.

8.2 VHL and Relevance to Skeletal Disease

A characteristic lesion found in VHL is the endolymphatic sac tumor (ELST), previously referred to as a low-grade adenocarcinoma of the temporal bone.[187,190] These are found in ~4% of patients with VHL and arise within the intraosseous portion of the middle ear and in particular the endolymphatic duct of the vestibular aqueduct. The ELST lesions are typically highly vascular, and although histologically characterized as benign, may display locally aggressive behavior with invasion and infiltration of the petrous bone leading to destruction of the labyrinth. Typically, patients present with tinnitus, vertigo, and sensorineural hearing loss, and surgical resection, if possible, is the treatment of choice with the aim of preserving hearing.

8.3 Genetics and Screening

The *VHL* gene, which is located on chromosome 3p26-p25 comprises three exons and encodes a 213 amino acid protein (pVHL) (Table 38.2).[188,189,191] Approximately 80% of patients inherit the mutation from an affected parent, while ~20% of cases arise de novo. Missense *VHL* mutations occur most commonly, with the remainder represented by loss-of-function mutations. VHL-associated tumors typically demonstrate biallelic inactivation of the *VHL* gene consistent with a tumor suppressor function, further supported by the high frequency of somatic *VHL*

TABLE 38.10 Genotype–Phenotype Correlations in Von Hippel–Lindau (VHL)

VHL subtypes	VHL mutation types	Clinical manifestations
1	Deletions, nonsense, frameshift, and missense	CNS/retinal hemangioblastoma, clear cell renal cell carcinoma
2A	Missense (e.g., Tyr112His)	CNS/retinal hemangioblastoma, pheochromocytoma
2B	Missense (e.g., Arg167Gly)	CNS/retinal hemangioblastoma, clear cell renal cell carcinoma, pheochromocytoma
2C	Missense (e.g., Leu188Val)	Pheochromocytoma

The majority of individuals with gene deletions or mutations resulting in premature protein truncation develop type 1 disease, whereas >80% of kindreds with type 2 disease harbor missense mutations, and the majority of these, the 2B variant.

inactivation in sporadic clear cell RCC (~80%) and hemangioblastomas (~50%).

pVHL is widely expressed and likely harbors tissue-specific activities. However, its clearest defined function is in the highly conserved oxygen-sensing pathway regulating hypoxia-inducible factor 1-α (HIF1α). In this pathway, under normoxic conditions, HIF1α undergoes hydroxylation by the proryl hydroxylases (PHDs), which enables capture by pVHL, and subsequent proteosomal degradation. In hypoxic condition, the reduced hydroxylation of HIF1α prevents VHL-mediated degradation, with activation of HIF1α target gene expression. In the presence of mutant (or absent) pVHL, aberrant stabilization of HIF1α occurs even under normoxic conditions, giving rise to inappropriate activation of the HIF1α pathway.[189] This promotes processes relevant to tumorigenesis, including the overproduction of vascular endothelial grow factor (VEGF) and angiogenesis. Genotype–phenotype correlations are reported in VHL (Table 38.10), and recent studies indicate that amino acid substitutions affecting the surface of the VHL protein are associated with a higher risk of pheochromocytoma.[188,189,191,192] Genetic testing for *VHL* mutations is recommended in a wide variety of clinical settings (Table 38.11), while screening for presymptomatic lesions is recommended.

8.4 Role of VHL in Bone Biology

Although skeletal manifestations are not a characteristic features of VHL with the exception of endolymphatic sac tumors, considerable interest has focused on the role of VHL and HIF1α signaling in bone biology, in part reflecting the temporal and spatial association between skeletal development and angiogenesis, and the role of the osteoblast in oxygen sensing. Studies of mouse models have shown that the *Vhl* plays an important homeostatic role in skeletal development.[193–195] Conventional *Vhl* knockout null mice ($Vhl^{-/-}$) demonstrate embryonic lethality due to aberrant placental angiogenesis,[196] and conditional knockout models have therefore been used to investigate the role of *Vhl* in skeletal lineages. Thus, depletion of *Vhl* in osteoblasts resulted in a phenotype characterized by dense, heavily vascularized long bones due to increased HIF1α expression, and upregulation of VEGF.[197] Consistent with these findings is the observation that pharmacologic activation of the HIF1α pathway using small molecule inhibitors increases vascularity and accelerated bone regeneration.[198] In contrast, *Vhl* depletion in chondrocytes results in severe dwarfism characterized by reduced chondrocyte proliferation and increased extracellular matrix.[193] Notably, a recent model in which *Vhl* is inactivated postnatally in osteochondral progenitor cells resulted in a similar phenotype to that observed in mice lacking *Vhl* in mature osteoblasts with progressive accumulation of cancellous bone and increased microvascular density,[195] while loss of *Vhl* in mesenchymal progenitor cells of the limb bud (i.e., prior to chondrocyte lineage commitment) resulted in marked defects in endochondral bone development with structural collapse of the growth plate.[194] These finding indicate that manipulation of Vhl/HIF1α signaling may represent a therapeutic target for bone-loss disorders, including osteoporosis, although the relevance of each of these findings to human skeletal health remains to be established.

TABLE 38.11 Potential Indications for Consideration of *VHL* Genetic Testing

Clinical contexts	
Single VHL associated manifestation	Retinal/CNS hemangioblastoma
	Pheochromocytoma
	Endolymphatic sac tumor
	Clear cell renal cell carcinoma <50 years[a]
>1 VHL-associated manifestation	Pancreatic neuroendocrine tumor
	Clear cell renal cell carcinoma >50 years[b]
	Pancreatic cystadenoma
	Epididymal/adnexal cystadenoma
Familial context	All first degree relatives of known *VHL* mutation carrier
	Asymptomatic or symptomatic first degree relatives of individual with history of VHL-associated tumors (i.e., unknown *VHL* mutation status)

[a]*Or diagnosed >50 years with a first-degree relative with clear cell renal cell carcinoma.*
[b]*Plus no first-degree relative with clear cell renal cell carcinoma.*

9 CARNEY COMPLEX

9.1 Clinical Findings and Treatment

CNC is an autosomal dominant disorder characterized by pigmented lesions affecting the skin and mucosa (usually of the face, labia, and conjunctiva); myxomas (typically the eyelids and heart); psammomatous melanotic schwannomas (usually of the sympathetic nerve chain and upper gastrointestinal tract); and endocrine tumors that involve the adrenals, sertoli cells, somatotrophs, thyroid, and ovary.[145,199] The clinical manifestations of CNC are diverse and highly variable, typically appearing over a number of years. These manifestations may become clinically apparent in early childhood, although the diagnosis may be delayed with a median age of detection of 30 years.

Cushing's syndrome, the result of primary pigmented nodular adrenal disease, is the most common endocrine manifestation of CNC and may occur in ~25%–60% of patients.[145] Patients with CNC and Cushing's syndrome

often have an atypical appearance in being thin. It is important to note that these patients may not have markedly increased levels of cortisol (e.g., in urine collections) and may demonstrate a paradoxical elevation in cortisol following dexamethasone.[145] In addition, abnormal cortisol production may only occur periodically (i.e., "periodic" or "cyclical" Cushing's syndrome). CNC patients with Cushing's syndrome usually have loss of the circadian rhythm of cortisol production.

Elevations of GH together with IGF-1, and/or PRL occur in ~75% of patients although typically these individuals are asymptomatic.[145,199–201] Clinical evidence of acromegaly due to an underlying pituitary somatotrophinoma is observed in ~10% of patients. Rare cases of prolactinoma have also been reported. Thyroid abnormalities are common with ~60% of patients harboring benign nodules. Rarely, papillary and follicular thyroid cancer have been reported. Testicular tumors may occur in one-third of male patients with CNC, while ovarian cysts and breast duct adenomas have been reported in female patients.

9.2 Carney Complex and Relevance to Skeletal Disease

A rare manifestation of CNC is that of osteochondromyxoma,[202] which typically occurs early in life (i.e., <2 years of age). These myxomatous tumors may affect any bone but are predominantly reported in the nasal sinuses and the long bones of the arms and legs. Although benign, local invasiveness, and recurrence of these tumors has been reported.[200] Individuals with CNC affected by Cushing's syndrome frequently manifest secondary osteoporosis as a consequence of hypercortisolemia.

9.3 Genetics and Screening

CNC1 is due to mutations of the protein kinase A (PKA) regulatory subunit 1 α(R1α) (PRKAR1A), a tumor suppressor, whose gene is located on chromosome 17q.24.2 (Table 38.2).[203] More than 100 heterozygous *PRKAR1A* mutations have been reported and the majority of these are unique occurring in single patients or kindreds.[145,199,200] The mutations are predicted to result in a loss of function with the majority predicted to result in premature truncation of the encoded protein. The majority of cases are inherited from an affected parent, although ~30% of cases may arise due to a de novo mutation. The gene causing CNC type 2 (CNC2) is located on chromosome 2p16, and has not yet been identified.[145,200]

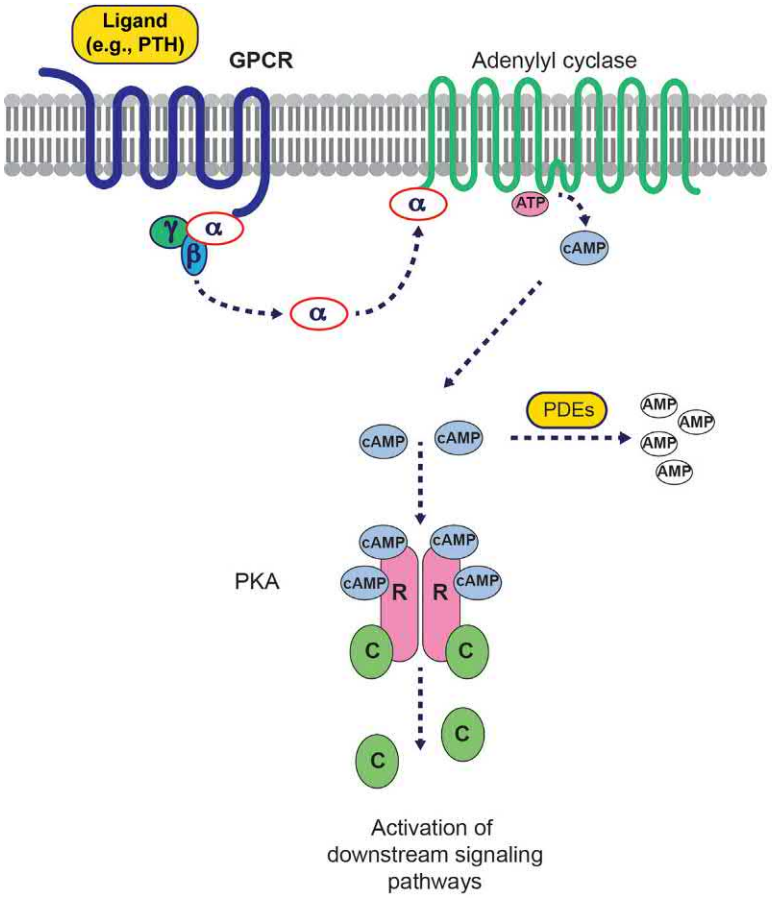
9.4 Role of cAMP-Protein Kinase A Signaling and *PRKAR1A* in Bone Biology

A key role for cAMP-PKA signaling during bone development is highlighted by the broad range of skeletal manifestations observed in individuals who harbor germline mutations in genes encoding key components of this pathway (Fig. 38.4). For example, polyostitic fibrous dysplasia occurs in individuals with MAS while multiple skeletal abnormalities are observed in Albright's Hereditary Osteodystrophy due to gain-of-function and loss-of-function *GNAS* mutations, respectively (Fig. 38.4). Inactivating mutations in *PRKAR1A* result in constitutive activation of the cAMP-PKA signaling pathway through the increased availability of PKA catalytic subunits.[200] Insights into the role of *PRKAR1A* in bone development have come from mouse models, for example, conventional haploinsufficient *Prkar1a* mice ($Prkar1a^{+/-}$) manifest a spectrum of abnormalities partially overlapping with those observed in Carney patients, including fibroosseous bone lesions.[204,205] Specifically, bony abnormalities of the tail vertebrae are observed in ~80% of $Prkar1a^{+/-}$ mice by 1 year of age, with the lesions demonstrating enhanced PKA activity and abnormal osteoblast differentiation with alterations in the Wnt signaling pathway.[205] Intercrossing $Prkar1a^{+/-}$ mice with $Tp53^{+/-}$ or $Rb^{+/-}$ mice demonstrates an enhanced tumorigenic effect[206] and $Prkar1a^{+/-}$ mice intercrossed with PKA catalytic subunit α haploinsufficient mice (i.e., $Prkaca^{+/-}$) also develop increased numbers of bone tumors, including those with features characteristic of osteochondromyxomas.[207,208] In addition, selective deletion of *Prkar1a* in cranial neural crest cells demonstrates defects in intramembranous ossification.[209] Finally, deletion of the *Prkar1a* locus is reported to constitute the causative genetic abnormality in an osteosarcoma mouse model, in which loss of *Prkar1a* results in PKA signaling deregulation, RANKL overexpression and accelerated osteosarcoma formation.[210] It is also noteworthy that a separate class of *PRKAR1A* mutation, in which the dissociation of the regulatory subunits from the catalytic subunits is inhibited, is observed in individuals with acrodysostosis (Fig. 38.4).[211,212] This disorder is characterized by a severe skeletal dysplasia consisting of brachydactyly, facial dysostosis, nasal hypoplasia, and short stature.[211,212]

10 COWDEN SYNDROME

10.1 Clinical Findings and Treatment

CWS is an autosomal dominant disorder with a prevalence ~1 in 200,000, that is characterized by the presence of multiple hamartomatous lesions, especially of the skin and mucous membranes (e.g., buccal, intestinal, and colonic) and an increased risk of developing specific cancers, including those affecting the breast, endometrium, and thyroid.[213,214] Thyroid abnormalities occur in



Gene	Gain-of-function Mutation	Loss-of-function Mutation
PTHR1	Jansens syndrome	Blomstrand syndrome
GNAS	MAS	PHP / AHO
PDE4D	—	Acrodysostosis[±]
PRKAR1A	Acrodysostosis[*]	Carney complex

FIGURE 38.4 Disruption of cAMP-protein kinase A (PKA) signaling in hereditary endocrine and skeletal disease. Several hormones, including parathyroid hormone (PTH) mediate their effects via activation of heterotrimeric G-protein-coupled receptors (GPCRs). Following ligand binding, the α-subunit of the stimulatory G-protein, encoded by *GNAS*, dissociates from beta (β) and gamma (γ) subunits to activate adenylyl cyclase with the resultant generation of cyclic AMP. Binding of cAMP to the regulatory (R) subunits of PKA, results in release of the catalytic (C) subunits, which facilitate serine–threonine phosphorylation of target proteins to activate downstream signaling pathways. Under physiological conditions, signaling through the pathway is regulated by a number of processes. For example, intrinsic inactivation of the α-subunit via GTP-to-GDP exchange and the degradation of cAMP by phosphodiesterases (PDEs) limit the availability of cAMP for signaling. Germline mutations affecting several components of cAMP/PKA pathway are associated with hereditary endocrine and skeletal disorders. For example, constitutive activating mutations in the GPCR *PTHR1* result in the autosomal dominant disorder Jansen chondrodysplasia, which is characterized by metaphyseal dysplasia. In contrast, Blomstrand chondrodysplasia, an autosomal recessive disorder due to inactivating mutations in the *PTHR1* gene, results in osteosclerosis, advanced skeletal maturation, and perinatal lethality. McCune–Albright Syndrome (MAS) results from gain-of-function mutations in *GNAS*, leading to constitutive activation of the α-subunit and enhanced cAMP production, while loss-of-function mutations in *GNAS* result in pseudohypoparathyroidism (PHP) and Albright hereditary osteodystophy (AHO), characterized by short stature, brachydactyly, rounded face, and ectopic ossification. Carney complex (CC), due to *PRKAR1A* loss-of-function mutations, results in defective regulatory (R) subunit activity with enhanced release of the catalytic subunits, and increased PKA signaling. In contrast, mutations in *PRKAR1A*, which result in an impaired ability of the regulatory subunits to dissociate from the catalytic subunits in response to cAMP (i.e., an enhanced inhibitory function, and thus a potential "gain-of-function"*), result in acrodysostosis, a disorder characterized by severe brachydactyly, facial dysostosis, nasal hypoplasia, and short stature. Acrodysostosis has also been reported in patients with germline mutations in the phosphodiesterase gene *PDE4D*, further highlighting the role of cAMP signaling in skeletal disease. However, the exact molecular mechanisms contributing to the disease phenotype in patients with *PDE4D* mutations remains to be determined, but likely reflects tissue-specific expression of PDE4D isoforms. Thus, although mutations in *PDE4D* are considered loss of function[±] with impaired enzymatic activity, enhanced cAMP signaling is not reported and may reflect compensatory upregulation of other PDE4D isoform expression.

two-thirds of patients with CWS, and these usually consist of multinodular goiter or benign adenomas, which may occur together with thyroiditis. The lifetime risk of thyroid cancer is ~10%–35%. Although both papillary and follicular thyroid neoplasms are reported, there is a significant overrepresentation of follicular neoplasms. Breast cancer is the most frequently reported malignancy with a lifetime risk of 25%–85%, together with a high prevalence of benign breast abnormalities.[215] Colonic polyps occur with high frequency and there is a likely increased risk of colonic cancer. Endometrial cancer is reported to be increased, as are renal cell carcinoma and melanoma. The investigation and treatment for CWS tumors is similar to that undertaken for non-CWS patients.

10.2 Cowden's Syndrome and Relevance to Skeletal Disease

Multiple vertebral hemangiomas leading to paraparesis and Ewing's sarcoma have been reported in a few CWS patients, and it seems that skeletal manifestations do not constitute a major component of CWS or related disorders.[216,217]

10.3 Genetics and Screening

CWS is genetically heterogeneous and seven types (CWS1-7) are recognized (Table 38.2).[215] Each is inherited as an autosomal dominant trait although penetrance of manifestations and clinical phenotypes may be variable. The most frequent genetic abnormality in individuals with CWS is mutation of the *phosphate and tensin homologue deleted on chromosome ten* (*PTEN*) gene, located on chromosome 10q23.31 and is refereed to as CWS1.[213,218] The frequency of *PTEN* mutations in cohorts of CWS patients ranges from 25%–85%. However, the phenotype of individuals with *PTEN* mutations are highly variable and as a consequence these are collectively referred to as the PTEN hamartoma tumor syndromes.[219] In patients with CWS but no *PTEN* mutation, a variety of alternate genetic etiologies may be involved (Table 38.2).[220] The variability of clinical phenotypes and incomplete penetrance may result in diagnostic challenges. The optimal screening protocol for individuals at risk of malignancy has not been established but should consist of regular evaluation for breast and thyroid abnormalities, as well as surveillance for endometrial, colonic, and dermatological tumors.

10.4 *PTEN* and Relevance to Skeletal Biology

Activation of PI3K with subsequent downstream stimulation of the Akt/Protein Kinase B signaling pathway plays an important role in bone development, and *PTEN* acts as key negative regulator of this pathway through its phosphatase activity, which inactivates the second messenger phosphatidylinositol (3,4,5)-triphosphate (PIP_3) to phosphatidylinositol-4,5-bisphosphate (PIP_2).[221] Several mouse models have investigated the regulatory role of *Pten* in bone development.[222–226] Conventional homozygous $Pten^{-/-}$ mice demonstrate early embryonic lethality, conditional deletion of *Pten* in osteoblasts leads to a dramatic and progressive increase in BMD throughout life as a result of rapid osteoblast differentiation and reduced apoptosis.[223] The absence of *Pten* in osteoblasts is also reported to result in improved intramembranous and late endochondral fracture healing,[225] while inactivation of *Pten* in osteochondroprogenitor cells results in increased skeletal size with enhanced trabeculation and cortical thickness and disruption to long bone growth plates, as a result of increased PI3K signaling.[222] Similarly, ablation of *Pten* from undifferentiated bone-destined mesenchymal cells, results in increased osteoblast numbers and enhanced bone matrix in surviving mice, mediated by increased fibroblast growth factor signaling.[224] More recent studies evaluating the consequences of codeletion of *Pten* and *Rb1* in osteoblast precursors, have reported that *Pten* deletion alone results in enhanced bone accumulation, consistent with earlier reports, and also increases adipocyte number within the bone marrow, and that *Pten* deletion combined with *Rb1* depletion results in adipogenic as opposed to osteogenic tumors.[226] Finally, somatic *PTEN* disruption is frequently observed in osteosarcoma formation, coupled with enhanced PI3K/Akt signaling, while functional loss of *PTEN* is reported in several tumors associated with bone metastases (e.g., prostate), multiple myeloma, and Ewing's sarcoma.[227] Together, such studies indicate that the PI3K/Akt/mTOR signaling may act as a potential therapeutic target in bone related malignancies.[227]

11 MCCUNE–ALBRIGHT SYNDROME

11.1 Clinical Findings and Treatment

MAS is characterized by the triad of polyostotic fibrous dysplasia, which may be associated with hypophosphatemia rickets, café-au-lait skin pigmentation, and peripheral precocious puberty, as well as other endocrine abnormalities that include thyrotoxicosis that may be associated with a multinodular goiter; somatotrophinomas; and Cushing's syndrome (due to adrenal tumors).[201,228] The treatment of each of these different clinical disorders in MAS patients is similar to that in non-MAS patients.

11.2 McCune–Albright Syndrome and Relevance to Skeletal Disease

Patients with MAS may have polyostotic fibrous dysplasia and hypophosphatemic rickets.[229] The fibrous dysplasia can involve a single bone (monostotic) or multiple bones (polyostotic) and the most frequent sites affected include the proximal femurs and skull base, although ribs, craniofacial bones, pelvis, and spine are also commonly affected.[201,228,229] However, the extent of disease is variable, and the sites of involvement are established early in life. Clinical manifestations often appear during rapid bone growth (i.e., during childhood and adolescence) and patients most frequently present with a limp, pain, or overt fracture. Pathological evaluation of fibrous dysplastic bone in patients with MAS demonstrates expansive fibroosseous lesions although site-specific histological features are observed.[201,228] Renal phosphate

wasting, with or without osteomalacia, is observed in patients with MAS and is reported to be due to the secretion of the phosphaturic hormone fibroblast growth factor 23 from the dysplastic tissue.[201,228] Treatment of fibrous dysplasia with bisphosphonates (e.g., pamidronate) has been suggested based on observational studies, which reported reduced pain, and potential improvements in radiographic appearances. However, a randomized controlled study of alendronate in fibrous dysplasia did not show significant improvements in these parameters.[228] Additional therapies, which may be of potential benefit for fibrous dysplasia, include the interleukin-6 receptor antagonist tocilizumab and antiRANKL antibody, denosumab.[229]

11.3 Genetics and Screening

MAS is caused by missense activating mutations of the *GNAS* gene that maps to chromosome 20q13, and encodes the alpha subunit of the stimulatory G-protein ($G_s\alpha$).[201] The primary function of $G_s\alpha$ is to couple ligand–receptor binding with cyclic AMP production by adenylyl cyclase (Fig. 38.4). The $G_s\alpha$ mutations observed in patients with MAS result in constitutive cAMP production (i.e., in the absence of ligand) as a result of impaired GTPase activity, and results in aberrant downstream signaling. Two amino acids are typically affected in MAS with ~95% of patients harboring mutations affecting residue Arg201 and ~5% affecting Gln227. The *GNAS* mutations associated with MAS are not inherited in the germline, but demonstrate somatic mosaicism consistent with their occurrence in early embryonic development.[201,228] The variability of the clinical phenotype observed in MAS is likely determined by the timing of *GNAS* somatic mutation and extent of mosaicism.

11.4 MAS and Relevance to Skeletal Biology

The tissue defects observed in MAS result from the constitutive activation of $G_s\alpha$ and the associated downstream signaling pathways. The spectrum of skeletal phenotypes observed in individuals with gain- and loss-of-function *GNAS* mutations (e.g., MAS and pseudohypoparathyroidism/Albright's hereditary osteodystrophy, respectively), as well as gain- and loss-of-function mutations in *PRKAR1A* (i.e., acrodystosis and CNC, respectively) indicate that $G_s\alpha$ and downstream cAMP/PKA signaling play critical roles in bone biology and skeletal development (Fig. 38.4). Insights into the skeletal phenotype in MAS have been established from mouse models. Thus, mice constitutively expressing the $G_s\alpha$ Arg201Cys mutation develop fibrous dysplasia in the postnatal period that is histologically indistinct from the human disease.[230] However, although osteoblast-specific expression of the $G_s\alpha$ Arg201Cys mutation results in high bone mass phenotype, it does not reproduce fibrous dysplasia, thereby indicating that the skeletal defects observed in patients with MAS are not due to defects in mature osteoblasts.[231] Indeed studies of skeletal progenitor cells indicate that fibrous dysplasia is likely due to impairment of osteoblast differentiation from mesenchymal progenitor cells,[232] and that such defects may result from abnormal modulation of the Wnt/β-catenin pathway.[233]

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SECTION 5

VITAMIN D AND RENAL DISORDERS

CHAPTER

39

Genetic Disorders Of Vitamin D Synthesis and Action

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1 INTRODUCTION

Vitamin D_3 is an inactive precursor, requiring two hydroxylation steps. First the parent molecule cholecalciferol is 25-hydroxylated in the liver to yield 25-hydroxyvitamin D_3 [$25(OH)D_3$] and then 1α-hydroxylated in the kidney to yield 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] or calcitriol), the active hormone (Fig. 39.1). A similar pathway takes place with vitamin D_2 obtained from plant sources. In subsequent text we will omit the subscript (written without a subscript connotes either D_3 or D_2) except when referring specifically to $1,25(OH)_2D_3$ (calcitriol), the active human hormone. $1,25(OH)_2D$ then binds to the vitamin D receptor (VDR) to mediate its biological actions. $1,25(OH)_2D$/VDR complexes regulate the expression of multiple target genes throughout the body, notably genes that regulate calcium and phosphate metabolism and that mediate mineralization of bone. In the absence of either $1,25(OH)_2D$ or a functional VDR, calcium absorption is impaired and bones are inadequately mineralized. When this occurs in children, rickets develops, when vitamin D deficiency occurs in adults, osteomalacia develops. The most common cause of rickets and osteomalacia worldwide is nutritional vitamin D deficiency, but three rare and very interesting genetic diseases also cause pediatric rickets and are the subject of this chapter.

Vitamin D–resistant rickets, now known to be caused by mutations in VDR, and referred to in this chapter as hereditary vitamin D–resistant rickets or HVDRR was first described in 1937.[1] 25-Hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase) deficiency was first described in 1961.[2] Both were characterized by infantile onset, severe hypocalcemia, and varying degrees of hypophosphatemia, but were distinguished by the unresponsiveness of HVDRR to treatment with vitamin D, whereas 1α-hydroxylase deficiency was responsive to high doses of vitamin D, leading to the terms "hereditary pseudovitamin D deficiency rickets" (PDDR),[2] "vitamin D dependency" because of its responsiveness to vitamin D,[3] or vitamin D–dependent rickets type I (VDDR I). Both of these and related diseases, and their corresponding mouse knockouts, have provided insights into the pathways of $1,25(OH)_2D$ synthesis and degradation and the mechanism of action of $1,25(OH)_2D$.

The critical enzyme in the synthesis of $1,25(OH)_2D$ from its circulating precursor, 25(OH)D, is 1α-hydroxylase. Mutations in this enzyme impair $1,25(OH)_2D$ synthesis, causing deficiency of the active hormone. By contrast, defects in the vitamin D 24-hydroxylase, the principal enzyme that inactivates 25(OH)D and $1,25(OH)_2D$, can cause hypercalcemia. A variety of mutations in the gene for VDR cause HVDRR, also known as vitamin D–dependent rickets type II (VDDR II). Both 1α-hydroxylase deficiency and HVDRR are rare autosomal recessive disorders characterized by hypocalcemia, secondary hyperparathyroidism, and early-onset severe rickets. A key diagnostic difference is that 1α-hydroxylase deficiency is characterized by extremely low–to-absent serum $1,25(OH)_2D$ levels, while HVDRR, characteristic of a target organ resistance disease, is distinguished by exceedingly elevated levels of $1,25(OH)_2D$. This chapter

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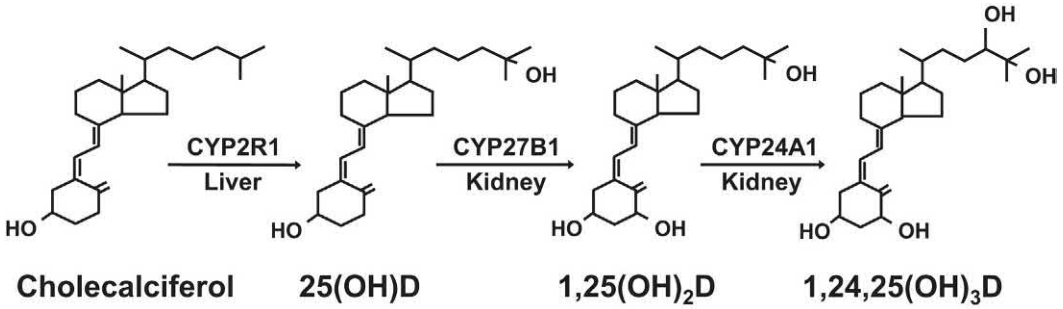

FIGURE 39.1 Ultraviolet light at 290–320 nm acts on 7-dehydrocholesterol in the skin to cleave its B-ring to yield cholecalciferol (vitamin D_3). Vitamin D may be converted to 25-hydroxyvitamin D [25(OH)D] by several enzymes in the liver, the principal one being CYP2R1 in the endoplasmic reticulum (ER). 25(OH)D is then activated through 1α-hydroxylation by mitochondrial CYP27B1 in the kidney, yielding the active hormone 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], which is the principal ligand for the vitamin D receptor (VDR). Both 25(OH)D and 1,25$(OH)_2$D may be inactivated via 24-hydroxylation by mitochondrial CYP24A1 to yield 24,25-dihydroxyvitamin D [24,25$(OH)_2$D] or 1,24,25-trihydroxyvitamin D [1,24,25$(OH)_3$D], respectively.

discusses the vitamin D biosynthetic enzymes and the VDR signaling molecule, focusing on the similarities and differences between 1α-hydroxylase deficiency and HVDRR, which present similarly in infancy.

2 BIOSYNTHESIS OF 1,25-DIHYDROXYVITAMIN D

2.1 Overview of Vitamin D Biosynthetic Enzymes

As with all steroid and sterol hormones, vitamin D, a secosteroid, is closely related to cholesterol. The final step in the biosynthesis of cholesterol is the conversion of 7-dehydrocholesterol to cholesterol by 7-hydroxycholesterol reductase (3β-hydroxysterol Δ7-reductase, DHCR7), the enzyme that is disordered in Smith–Lemli–Opitz syndrome (SLO, OMIM 270400).[4] Patients with this syndrome, DHCR7-null mice, and animals given inhibitors of DHCR7 have augmented vitamin D synthesis.[5] In human skin, ultraviolet radiation at 270–290 nm can directly cleave the 9–10 carbon–carbon bond of the cholesterol B ring, converting 7-dehydrocholesterol to cholecalciferol (vitamin D_3).[6] Plants produce a closely related sterol, ergocalciferol (vitamin D_2), that has nearly the same properties as cholecalciferol. Both forms of vitamin D are biologically inactive prohormones that are then activated, and subsequently inactivated, by a series of cytochrome P450 enzymes.

Cytochrome P450 refers to multiple enzymes that contain a heme group and absorb light at 450 nm in their reduced states; the human genome encodes only 57 such enzymes, but these catalyze thousands of reactions.[7] P450 enzymes may be found in mitochondria (Type I P450 enzymes) and in endoplasmic reticulum (ER) (Type II P450 enzymes), but all act as oxidases by receiving electrons from the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). In mitochondria, NADPH donates the electrons to a membrane-bound flavoprotein termed adrenodoxin reductase, which then donates them to a soluble iron–sulfur protein termed ferredoxin, which then donates them to the Type I P450; the vitamin D 1α-hydroxylase and 24-hydroxylase are Type I P450 enzymes. In the ER, NADPH donates electrons to a diflavin protein termed P450 oxidoreductase (POR), which then donates them to the Type II P450. This is a complex process in which the flavin adenine dinucleotide (FAD) group of POR accepts electrons from NADPH, eliciting a conformational change in the POR protein, so that the FAD becomes aligned with the flavin mononucleotide (FMN) moiety, which receives the electrons, permitting the POR to return to its original conformation, and thus permitting the interaction of the FMN domain of POR with the Type I P450.[8] The principal vitamin D 25-hydroxylase, as well as most hepatic drug-metabolizing enzymes, are Type I P450 enzymes; in some cases, cytochrome b5 acts as an allosteric factor to facilitate electron flow from POR to the Type I P450. The heme iron in both types of P450 ultimately receives the electrons and donates them to molecular oxygen as the terminal electron acceptor, mediating catalysis.

2.2 Vitamin D 25-Hydroxylases

The 25-hydroxylation of vitamin D primarily occurs in the liver, which contains several enzymes that can catalyze this reaction. Hepatic 25-hydroxylation appears to be passive; physiologic regulation of this step has not been demonstrated, and circulating concentrations of 25(OH)D are determined primarily by dietary intake of vitamin D and exposure to sunlight. Early cell fractionation and biochemical studies found 25-hydroxylase activity in both mitochondria and microsomal fractions. Screening of rat liver cDNA expression libraries with polyclonal antisera raised against a purified rat liver 25-hydroxylase preparation yielded cDNA for an enzyme then called

P450c25.[9,10] That enzyme could hydroxylate sterol carbons 26 and 27 to initiate bile acid synthesis, and is now referred to as P450c27 or CYP27A1.[11] The subsequent cloning of the mitochondrial 1α- and 24-hydroxylases showed that CYP27A1 was structurally related to these enzymes, suggesting that CYP27A1 might be a major vitamin D 25-hydroxylase. However, patients with CYP27A1 mutations have cerebrotendinous xanthomatosis without a disorder in calcium metabolism,[12,13] suggesting that at least one other enzyme besides CYP27A1 must also catalyze vitamin D 25-hydroxylation. Several other hepatic P450 enzymes were shown to catalyze 25-hydroxylase activity in vitro, including CYP2D25, CYP2D6, CYP3A4, CYP2C11, and CYP2J3, but their enzymology does not coincide well with the observed enzymology of human 25-hydroxylase.[14–18]

For most enzymes, there are corresponding rare genetic deficiency disorders, typically characterized by accumulation of the enzyme's substrate and lack of its product. However, very few patients had been described having apparent 25-hydroxylase deficiency, and there are no clear criteria for this potential diagnosis. The best clinical candidates were two Nigerian brothers who had hypophosphatemia, minimal hypocalcemia, grossly elevated alkaline phosphatase, normal serum $1,25(OH)_2D$, low 25(OH)D, and showed decreased intestinal vitamin D absorption.[19] A Saudi family appeared to be clinically and hormonally similar.[20] However, the genes for CYP27A1 and another candidate, CYP2D6, lacked mutations in these families.[21] A microsomal P450 enzyme, CYP2R1 was then identified as a vitamin D 25-hydroxylase that had higher affinity for vitamin D than did CYP27A1.[22] The homozygous CYP2R1 mutation L99P was found in an EBV-transformed cell line established from one of the original Nigerian patients, and this mutation dramatically reduced 25-hydroxylase activity when assayed in vitro.[23] Others confirmed the genetic findings in the Nigerian family, but found no mutations in the Saudi family.[24] CYP2R1 is widely expressed, possibly accounting for the persistent vitamin D 25-hydroxylation in patients with liver failure.[25] Studies of additional Nigerian families found no CYP2R1 mutations in 27 children with sporadic rickets, but found 3 individuals in 1 family who were homozygous for the previously identified L99P mutation and 1 individual in another family who was heterozygous for the novel mutation K242N.[26] Another study found two Saudi siblings with classic symptoms of vitamin D deficiency who were compound heterozygotes for the CYP2R1 mutations 367 + 1, G→A and 768, iT, both of which encode null alleles.[27] Thus, even though the combined knockout of both mouse Cyp2r1 and Cyp27a1 only reduced levels of 25(OH)D by 50%, did not affect circulating $1,25(OH)_2D$, and caused no obvious phenotype,[28] human genetic studies confirm a central role for CYP2R1 in human hepatic 25-hydroxylation of vitamin D.

Although CYP2R1 is the principal human vitamin D 25-hydroxylase, its deficiency is exquisitely rare, suggesting that both CYP2R1 and one or more other enzymes are effective 25-hydroxylases in vivo, so that symptomatic disease is only seen when there is a CYP2R1 mutation in the presence of another stressor, such as neonatal hypoparathyroidism or nutritional D deficiency. Genome-wide association studies have found polymorphisms in or near the *CYP2R1* gene associated with circulating concentrations of 25(OH)D,[29–34] and that such polymorphisms can modify responses to oral vitamin D supplementation,[35,36] possibly by influencing DNA methylation.[36]

CYP2R1 is highly specific for vitamin D 25-hydroxylation, even though it belongs to a family of hepatic drug-metabolizing enzymes that have broad substrate specificity. The crystal structure of CYP2R1 with vitamin D_3 bound in its catalytic site shows a typical microsomal cytochrome P450 structure, but with a more closed, tight conformation and with hydrophobic residues lining the substrate-binding pocket, so that the geometry is only suited to binding planar hydrophobic molecules, such as sterols.[37] The L99 residue mutated in the Nigerian families is located in the β-helix, near to, but not directly involved in binding vitamin D.

2.3 Vitamin D 24-Hydroxylase

Calcitriol may be inactivated by the principal hepatic drug-metabolizing enzyme, microsomal CYP3A4 in liver, but one of the more important mechanisms by which vitamin D can be inactivated is by its 24-hydroxylation by 25-hydroxyvitamin D-24-hydroxylase (P450c24, CYP24A1). This mitochondrial enzyme can catalyze the 24-hydroxylation of 25(OH)D to $24,25(OH)_2D$ and of $1,25(OH)_2D$ to $1,24,25(OH)_3D$, primarily in the kidney and intestine[38–40]; both reactions initiate the inactivation pathway of vitamin D leading to calcitroic acid, although some evidence suggests certain activities for these 24-hydroxylated compounds. P450c24 can also catalyze 23-hydroxylation of 25(OH)D and $1,25(OH)_2D$ to $23,25(OH)_2D$ and $1,23,25(OH)_3D$.[41] The $1,25(OH)_2D$-26,23-lactone produced via the C-23 pathway may inhibit VDR.[42,43] P450c24 was cloned by purifying the protein from rat renal mitochondria, raising a polyclonal antiserum, and screening a rat kidney cDNA expression library.[44] The human cDNA[40] and gene[45] were cloned soon thereafter. P450c24 is induced by $1,25(OH)_2D$, thus favoring its inactivation by 24-hydroxylation as a mechanism to regulate the amount of available $1,25(OH)_2D$.[46] The crystal structure of rat P450c24 shows a classic cytochrome P450 fold with an open conformation, despite which it exhibits relatively narrow substrate specificity, like other mitochondrial P450s.[47]

Whereas defects in CYP27B1 cause hypocalcemia, defects in CYP24A1, first described in 2011, can cause hypercalcemia.[48,49] The initial patients were infants less than 1-year old exhibiting weight loss, failure to thrive, hypercalcemia, hypercalciuria, and/or nephrocalcinosis, normal 25(OH)D levels, normal-to-moderately elevated $1,25(OH)_2D$ levels, low $24,25(OH)_2D$ levels, and low parathyroid hormone (PTH); most were also receiving vitamin D supplementation before diagnosis. More than 20 patients with hypercalcemia and CYP24A1 mutations have been reported.[48–56] Many of the apparently responsible CYP24A1 mutations have not been characterized functionally, as conventional mass spectrometric assays require complex equipment and expensive reagents, but a simple genetic assay may obviate this difficulty.[56]

Infantile hypercalcemia is associated with anorexia, emesis, lethargy, constipation, polyuria, irritability, seizures, and mental retardation. When other diagnoses, such as Williams–Beuren syndrome, calcium-sensing receptor mutations, neonatal familial hyperparathyroidism, thyroid disease, elevated parathyroid hormone related protein (PTHrP), Jansen's metaphyseal dysplasia, subcutaneous fat necrosis, phosphate depletion, and vitamin A or D toxicity, are excluded, the diagnosis has traditionally been "idiopathic infantile hypercalcemia." Many of these infants appear to harbor mutations of CYP24A1. The initially described patients carried homozygous or compound heterozygous mutations, but heterozygosity for CYP24A1 mutations may lead to clinically apparent hypercalcemia in the setting of excessive intake of vitamin D. A sharp rise in the incidence of idiopathic infantile hypercalcemia in the United Kingdom in the mid-1950s was temporally associated with excessive fortification of milk and cereal with vitamin D, so that infants ingested about 4000 units/day.[57] It has been speculated that these infants may have been heterozygous for CYP24A1 mutations, as such manifesting heterozygotes have been documented in adults receiving excessive supplements of vitamin D.[51,56]

2.4 Vitamin D 1α-Hydroxylase

The active, hormonal form of vitamin D, $1,25(OH)_2D$, is produced by the 1α-hydroxylation of 25(OH)D by a mitochondrial 1α-hydroxylase variously termed 25-hydroxyvitamin D-1α-hydroxylase, P450c1α, or CYP27B1 (Fig. 39.1). $1,25(OH)_2D$ in the circulation primarily reflects its renal synthesis, but 1α-hydroxylase activity is also found in keratinocytes, macrophages, osteoblasts, and placenta.[58–61] CYP27B1 has been found in many tissues containing the VDR, including breast, prostate, and colon, as well as the tumors and cancers that develop in these tissues. 1α-Hydroxylation is the rate-limiting step in the bioactivation of vitamin D, and renal enzyme activity is tightly regulated by PTH, calcium, phosphorus, and $1,25(OH)_2D$ itself.[62] The enzyme catalyzing 1α-hydroxylation has been of long-standing interest because of its importance in normal physiology and because $1,25(OH)_2D$ synthesis is impaired in several disorders, including chronic renal insufficiency, Fanconi syndrome, X-linked hypophosphatemic rickets, and VDDR I.

Efforts to purify the renal 1α-hydroxylase enzyme were unsuccessful, primarily because there is very little of this protein in renal mitochondria, hence immunologic approaches could not be used to clone the 1α-hydroxylase, as had been done for the 24- and 25-hydroxylases. However, in the second half of 1997, four independent groups using different approaches reported the cloning of the human, rat, and mouse vitamin D-1α-hydroxylase cDNAs[63–67] and the human gene,[64,68] subsequently termed *CYP27B1*. One group used mice with a knocked-out VDR to induce overproduction of 1α-hydroxylase and then screened a cDNA expression library for activation of a VDR construct.[67] Two other groups enriched renal 1α-hydroxylase mRNA by feeding rats a diet low in calcium and phosphorus, then used probes related to the conserved P450 heme-binding site to identify candidate sequences.[65,66]

The first human clone[63] was obtained by using RNA prepared from primary cultures of human keratinocytes, which, when grown in serum-free, low-calcium medium, have substantial 1α-hydroxylase activity.[60] Screening the keratinocyte cDNA library with oligonucleotides based on the relatively well-conserved sequences of the ferredoxin- and heme-binding sites of other P450s ultimately yielded the full-length human P450c1α cDNA.[63] Although the P450c1α cDNA was cloned from human keratinocytes, four lines of evidence demonstrated that the keratinocyte and renal P450c1α enzymes are encoded by the same gene. First, when the keratinocyte P450c1α cDNA was transfected into mouse Leydig MA-10 cells, the transfected cells catalyzed the conversion of 25(OH)D to authentic $1,25(OH)_2D$, as determined by high-pressure liquid chromatography and by gas chromatography/mass spectrometry of the 1α-hydroxylated product. Second, the cloned P450c1α had a Michaelis constant (K_m) of 2.7×10^{-7} M for 25(OH)D, which closely approximates the concentration of 25(OH)D found in vivo. Third, reverse transcription/polymerase chain reaction showed that identical P450c1α sequences are found in human keratinocytes and kidney. Finally, keratinocyte cDNA from a patient with VDDR I carried mutations in the cloned sequence, and analysis of the patient's genomic DNA revealed the same mutations. These findings provided genetic proof of the identify of the P450c1α and the first proof that VDDR I is caused by mutations in the gene encoding vitamin D-1α-hydroxylase.[63]

Shortly after the P450c1α cDNAs were cloned, the human gene was cloned,[64,68] localized to chromosome 12 by

somatic cell hybrid analysis,[68] and mapped to 12q13.1-13.3 by fluorescence in situ hybridization.[66,69,70] The human gene for 1α-hydroxylase is only 5 kb in length, is single copy, and comprises nine exons and eight introns.[68] Although it is substantially smaller than the genes for other mitochondrial P450 enzymes, its intron/exon organization is very similar, especially to that of the cholesterol side-chain cleavage enzyme, P450scc.[68] This strongly suggests that although the mitochondrial P450 enzymes retain only 30%–40% amino acid sequence identity with each other, they all belong to a single evolutionary lineage.

2.5 1α-Hydroxylase Deficiency

The disease previously called PDDR, VDDR, or VDDR I is now known to be caused by mutations in the *CYP27B1* gene, which encodes the enzyme that converts 25(OH)D to $1,25(OH)_2D$,[63] hence this disorder is more appropriately termed vitamin D 1α-hydroxylase deficiency.[71] Patients with 1α-hydroxylase deficiency are normal at birth and typically come to medical attention between 1 and 2 years of age with growth retardation, poor gross motor development, and generalized muscle weakness. Some infants are irritable when held, presumably due to bone pain, or develop pneumonia or hypocalcemic seizures. Physical findings resemble those seen in dietary vitamin D deficiency: enlarged costochondral junctions of the ribs (rachitic rosary), metaphyseal flaring of the wrists or ankles, genu varus, and occasionally, hypotonia, frontal bossing, enlarged sutures and fontanelles, or craniotabes (softening of the parieto–occipital area). Muscle traction on the softened rib cage can give rise to thoracic deformity, including pectus carinatum. Delayed dental eruption, enamel hypoplasia, and early caries are common. The long bones show the typical radiographic abnormalities of rickets: widening of the metaphysis, fraying, cupping and widening of the zone of provisional calcification, and diffuse demineralization. The chest X-ray may show the rachitic rosary and children old enough to stand and bear weight may have bowed tibias and femurs.

The clinical chemistry findings are not diagnostic, as they resemble those in patients with vitamin D deficiency rickets: hypocalcemia, hypophosphatemia, increased serum alkaline phosphatase activity, and increased serum PTH.[72–74] Calcium and phosphorus are not absorbed appropriately by the gut, and urinary calcium excretion is reduced.[73,75] As in patients with vitamin D deficiency, there may be hyperchloremic metabolic acidosis and generalized hyperaminoaciduria.[72,73,75]

Patients with 1α-hydroxylase deficiency have greatly reduced serum concentrations of $1,25(OH)_2D$ despite normal concentrations of 25(OH)D, and their clinical and laboratory abnormalities respond to physiologic replacement doses of $1,25(OH)_2D_3$.[3,74,76,77] By contrast, patients with nutritional vitamin D deficiency have reduced concentrations of 25(OH)D, and patients with HVDRR have greatly increased concentrations of $1,25(OH)_2D$. Some patients with genetically proven 1α-hydroxylase deficiency have serum concentrations of $1,25(OH)_2D$ in the normal range,[77] but such values are inappropriately low for the low serum calcium and phosphorus and the increased PTH, which should all increase production of $1,25(OH)_2D$. Patients with 1α-hydroxylase deficiency have normal concentrations of $24,25(OH)_2D$.[78] A comparison of the findings in 1α-hydroxylase deficiency and HVDRR is shown in Table 39.1.

2.6 *CYP27B1* Mutations Causing 1α-Hydroxylase Deficiency

1α-Hydroxylase deficiency is rare in most populations, but may be common in isolated populations as a result of founder effects. Among French Canadians in the Charlevoix–Saguenay–Lac Saint Jean area of Quebec the carrier rate is 1 in 26, so that the incidence of affected individuals is 1 in 2700 in this population.[79] This high incidence in French Canadian families permitted genetic mapping of 1α-hydroxylase deficiency to chromosome

TABLE 39.1 Comparison of the Defects in Affected Cases of 1α-Hydroxylase Deficiency and Hereditary Vitamin D–Resistant Rickets (HVDRR)

Characteristics	1α-Hydroxylase deficiency	HVDRR
Mutated gene	*CYP27B1*	*VDR*
Serum $1,25(OH)_2D_3$ levels	Low to absent	High
Serum PTH levels	High	High
Serum calcium levels	Low	Low
Serum phosphate levels	Low	Low
Alopecia	No	Yes
Usual response to calcitriol	Yes	No

12q14.[80] Microsatellite haplotyping distinguished two distinct founder populations in Canada: one from the Charlevoix–Saguenay–Lac Saint Jean region of Quebec and the other from eastern (Acadian) Canada.[81] The cloning of the 1α-hydroxylase gene has permitted molecular genetic analysis of 1α-hydroxylase deficiency in many patients from around the world. Well over 100 genetically confirmed cases of 1α-hydroxylase deficiency, involving about 50 different mutations in the *CYP27B1* gene have been reported.[63,69,77,82–93]

As 1α-hydroxylase deficiency is rare, most studies have described only a few patients or a single ethnic group. However, Wang et al.[84] identified the mutations in 19 patients from 17 families, including 5 French Canadian, 3 Polish, 4 Caucasian American, 1 Filipino, 1 Chinese, 1 Haitian, 1 African American, and 1 Hispanic family. Each patient was healthy at birth but came to medical attention within the first 2 years of life, typically with growth retardation or poor gross motor development. All had hypocalcemia, hypophosphatemia, increased serum alkaline phosphatase and PTH, normal serum 25(OH)D, and low or undetectable concentrations of $1,25(OH)_2$ D. All had radiographic evidence of rickets and all responded to physiologic replacement doses of $1,25(OH)_2D_3$. Microsatellite haplotyping and DNA sequencing showed that French Canadian patients from the Charlevoix region all carried a single haplotype and the same *CYP27B1* mutation that deletes guanosine 958 in codon 88, thus changing the reading frame and creating a premature stop codon, so that there could be no enzyme activity. This mutation destroys the sequence recognized by the endonucleases *Tai*I and *Mae*II, permitting rapid, accurate polymerase chain reaction–based diagnosis in this commonly affected population.[84] This study also found a 7-bp duplication on seven alleles in six families, but this mutation was associated with several different microsatellite haplotypes and was found in several unrelated ethnic groups (Polish, Chinese, Hispanic, Filipino, Caucasian American, and African American) indicating that the 7-bp duplication arose de novo among many different ethnic groups.[84] Others have found the same result in smaller groups of patients.[70,82]

Among the 14 mutations identified by Wang et al.,[84] seven changed single amino acids (missense mutations), but patients carrying the missense mutations or the frameshift/stop codon mutation could not be distinguished clinically. To assess their enzymatic activity, each missense mutant was recreated in vitro and expressed in mouse Leydig MA-10 cells, which were chosen because they were steroidogenic, and hence had ample ferredoxin and adrenodoxin reductase needed by Type I P450 enzymes, such as P450c1α. In these cells, none of the mutant proteins had measurable 1α-hydroxylase activity.[77] Other early studies that included patients with mild clinical abnormalities also failed to find partial activity of P450c1α missense mutants in a VDR activation assay[69,83] or in peripheral blood macrophages from other patients.[82] A recent study of 10 patients found only 8 different mutations, including 4 that had been described previously; this study included 5 patients from Korea, among whom 9 of 10 alleles carried only 2 mutations, indicating founder effects in this population.[85]

A few P450c1α mutations have been described that retain partial activity and cause mild disease. A mutation (E189G) that retained 22% of normal activity in vitro, caused mild disease: hypotonia, leg deformity, and secondary hyperparathyroidism, but with normal serum calcium, phosphorus, and $1,25(OH)_2D$.[84] The diagnosis of 1α-hydroxylase deficiency was suggested by unresponsiveness to large doses of vitamin D_3 but rapid response to 0.25 μg/day of $1,25(OH)_2D_3$. In another patient whose mutation (L343F) retained 2.3% of wild-type (WT) activity,[84] serum concentrations of 25(OH)D and $1,25(OH)_2D$ were not reduced, but serum phosphorus, alkaline phosphatase, and PTH were high. Another mild missense mutation (G102E) that retained about 20% of normal activity in vitro was found in six related Saudi individuals.[86] Nevertheless, there is considerable phenotypic variation among patients with 1α-hydroxylase deficiency who have mutations lacking assayable activity; the basis of this poor correlation of the clinical findings with the activities of the mutant P450c1α enzymes in vitro remains unclear. Thus, the classical laboratory criteria for the diagnosis of 1α-hydroxylase deficiency may not identify patients with partial enzymatic defects, hence 1α-hydroxylase deficiency may be more common than is generally appreciated.

Several Chinese and Turkish patients have been reported recently. Among eight Chinese families there were six novel missense mutations (G57V, G73W, L333F, R432C, R459C, and R492W), and three novel deletion mutants (c48-60del, c1310delG, and c1446delA); one allele also carried the recurrent 7-bp mutation discussed earlier. The missense mutants retained 5.5%–12.1% of WT activity.[89] Another Chinese patient was compound heterozygous for an 11-base deletion (c.311-321delGGCCCGAGCGC) and the missense mutation L158P.[88] Another patient was compound heterozygous for the novel missense mutations R389C and R459C, but the functional enzymology of these mutations was not assessed.[91] Among four Turkish families with seven affected patients, one was compound heterozygous for R389H and a novel nonsense mutation c.1079 C>A, another family had two persons homozygous for the novel splice donor site mutation c.195 + 2 T>G, and the other two families had four persons homozygous for the recurrent 7-bp insertion. Interestingly, one of these who had severe hypocalcemia and seizures at 4 months improved spontaneously in later childhood and had normal

serum calcium and $1,25(OH)_2D$ after being untreated for 12 months at age 11.[90] Another study of eight patients from seven apparently unrelated Turkish families found a novel splice donor site mutation (c.1215 + 2 T>A), which caused skipping of exon 7, two novel frameshift mutations: a 16-bp deletion (c.1022-1037del16) and a 2-bp deletion (c.934_935delAC).[92]

Whether or not variations in the *CYP27B1* gene contribute to other diseases remains unclear. In a study of 153 men and 596 women over 65-years old, a single-nucleotide polymorphism in the promoter of *CYP27B1* appeared to be associated with an increased risk for fractures, but this polymorphism had no effect on transcription of a promoter/reporter construct in vitro.[94] A study of >58,000 sequence variations in 43 patients with multiple sclerosis (MS) reported an association with the R389H mutation in *CYP27B1*,[95] which is an established cause of 1α-hydroxylase deficiency.[77,85] These authors also found heterozygosity for the *CYP27B1* mutations E189G and L343F in a second cohort of patients with MS, but to date none of the studies directly assessing 1α-hydroxylase deficiency have reported MS in their subjects. Thus it is not clear whether variations in *CYP27B1* directly contribute to neurologic disease.

The tertiary structures of several Type I P450 enzymes have been determined recently, including rat P450c24[47] and human P450scc.[96,97] These structures are remarkably similar despite their low amino acid sequence identity. As of mid-2016, a crystal structure has not been reported for P450c1α from any species. Linear sequence alignments of P450c1α permit logical deductions about how each P450c1α mutation impairs catalysis and results in disease.[77,98] A three-dimensional model of human P450c1α has been reported,[99] but this model is based on the crystal structure of rabbit CYP2C5, a Type II P450. Nevertheless, this model appeared to predict the activities of several mutations causing 1α-hydroxylase deficiency and predicted that T409 is essential for catalytic activity by hydrogen bonding with the 25(OH) group of $1,25(OH)_2D$.[100,101]

2.7 Treatment of 1α-Hydroxylase Deficiency

Three approaches have been tried to treat 1α-hydroxylase deficiency. The first was the use of massive doses of 50,000–200,000 units/day of vitamin D_2, which partially improved the clinical, chemical, and radiographic abnormalities. The basis of this partial efficacy is unclear; it is possible that high concentrations of forms of vitamin D that have not been 1α-hydroxylated might bind to and transactivate the VDR.[102–104] Alternatively, enzymes other than P450c1α may have low levels of "gratuitous" 1α-hydroxylase activity.

The second approach was to use forms of vitamin D that could bypass the defect in P450c1α. Two such agents have been used: dihydrotachysterol, which was widely used in the United States, and $1\alpha(OH)D_3$ (the 1α-hydroxylated form of vitamin D), which was used in Europe. Dihydrotachysterol, a synthetic 25-hydroxylated analog of $1,25(OH)_2D$, is highly effective at 50 mg/kg/day in infancy or 0.5–1.0 mg/day in adults. It is hydroxylated at the 3α-position instead of the 1α-position; rotation of the A-ring about the 6–7 carbon bond that connects to the C-ring brings this group into a position similar to the authentic 1α-position, permitting receptor binding and transactivation. $1\alpha(OH)D_3$ only requires hepatic 25-hydroxylation to become fully active. Doses of 1–3 μg/day (80–100 ng/kg) of $1\alpha(OH)D_3$ readily reversed the hypocalcemia, hyperparathyroidism, and rickets of 1α-hydroxylase deficiency.[105]

The third, and currently most popular current treatment of 1α-hydroxylase deficiency uses physiologic replacement doses of $1,25(OH)_2D_3$ (0.25–2.0 μg/day; 10–400 ng/kg/day). Calcitriol is the most potent and most rapidly acting form of vitamin D. Oral therapy rapidly corrects the hypocalcemia, secondary hyperparathyroidism, and rickets; restores bone mineral content; and repairs bone architecture.[74] Higher doses are initially used to induce healing of rickets, followed by lower maintenance doses; therapy must be life long, is predictably successful,[77] and restores bone mineral density.[87,93] Irrespective of the form of vitamin D used, one must monitor serum calcium, phosphorus, and PTH at regular intervals. Calcium intake must be substantial during the initial phase of therapy when bone healing consumes robust amounts of calcium (hungry bone syndrome). One generally aims to increase the total serum calcium concentration into the low–normal range (8.5–9 mg/dL), so as to suppress PTH to values just below the upper limit of normal; higher calcium concentrations increase the risk of hypercalciuria and nephrocalcinosis. The excretion of urinary calcium should be monitored, and the ratio of urinary calcium to urinary creatinine should remain less than 0.25; the 24-h urinary excretion of calcium should remain below 4 mg/kg. Long-term follow-up (up to 26 years) of 39 patients in Quebec, most of whom carried the "Charlevoix" mutation, supports this approach.[87] The most common presenting features among 21 patients under 2.6 years were rickets, motor delay, inability to walk, and short statute; treatment with calcitriol normalized hormonal and chemical values and lumbar bone mineral density within 3 months; catch-up growth took about 2 years. Among adult patients, final height correlated with the age at which treatment was started. Thus the outcome is excellent when treatment is begun in early childhood.

2.8 Other Pathways of Vitamin D Metabolism

In the synthesis of conventional steroid hormones in the adrenal, gonad, and placenta, the first and

rate-limiting step is the conversion of cholesterol to pregnenolone by P450scc, a mitochondrial enzyme encoded by the *CYP11A1* gene.[106,107] This conversion entails sequential 20-hydroxylation, 22R-hydroxylation, and subsequent cleavage of the 20–22 carbon bond on the side chain of cholesterol. The side chain of cholesterol is retained in vitamin D, 25(OH)D, and $1,25(OH)_2D$, and P450scc and its cofactors are found in skin.[108] In vitro, P450scc can convert vitamin D_3 to 20(OH)D and $20,22(OH)_2D$.[109] In vivo, P450scc produces many metabolites of vitamin D, principally 20(OH)D, which is found in human serum at about 3 nM, or 5% of the levels of 25(OH)D.[110,111] 20(OH)D lacks the calcemic activity of $1,25(OH)_2D$, but physiologic concentrations of 20(OH)D have VDR agonist activity that promotes antiproliferative, antiinflammatory, antifibrogenic, and prodifferentiative activity and protects DNA from damage by UV radiation.[112–115] Thus 20(OH)D may have useful therapeutic properties.

3 HEREDITARY VITAMIN D–RESISTANT RICKETS

3.1 Structure of the Vitamin D Receptor

The overall structure of the VDR protein is similar to the other members of the steroid–thyroid–retinoid receptor superfamily.[116] Elucidation of the etiology of naturally occurring mutations in children with HVDRR,[117–120] as well as designed mutations in the VDR, have helped to understand the functional domains of the VDR protein (Fig. 39.2).[121–130] The N-terminus of the VDR contains a highly conserved DNA-binding domain (DBD) while the C-terminal half of the protein harbors a more variable ligand-binding domain (LBD). The DBD contains two finger-like structures of 12–13 amino acids each. Four cysteine residues bind one zinc atom to form each zinc finger structure.[131] Regions of the DBD are not only critical for DNA binding, but also for mediating heterodimerization with the retinoid X receptor (RXR).[132,133] The hinge region (amino acid residues 93–120) connects the DBD and LBD and contains some of the nuclear localization signals.[134]

The VDR LBD is formed by amino acids 123–427. X-ray crystallography of the VDR showed that the LBD is composed of 12 α-helices (H1–H12) and 3 β-sheets (S1–S3).[135]

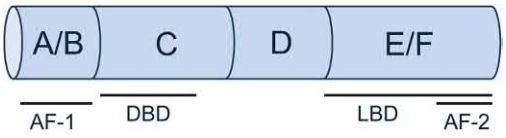

FIGURE 39.2 Structure of the human VDR with different domains depicted as A/B, C, D, and E/F. *AF*, Activating function; *DBD*, DNA-binding domain; *LBD*, ligand-binding domain.

Helix H12 forms a retractable lid that traps and holds the ligand in position. Ligand binding causes a conformational change in the VDR that promotes heterodimerization with RXR. VDR heterodimerization with RXR involves residues in H9, H10, and an E1 domain that overlaps H4 and H5 within the LBD. An activating function domain 2 (AF-2 domain) at residues 416–424 of helix H12 and the region between amino acids 232–272 encompassing H3 and H4 are essential for transactivation.[135] Repositioning of helix H12 after ligand binding is critical for the formation of a hydrophobic groove that binds LXXLL motifs (where L is leucine and X is any amino acid) in the nuclear receptor–interacting domains of coactivators. Other regions of the VDR also recruit coactivator proteins or facilitate contact with proteins associated with the core transcriptional machinery, such as TFIIB or the TAFs.[136,137] Heterogeneous mutations in the VDR LBD leading to HVDRR caused different effects on ligand or RXR binding, which vary from completely abolished ligand binding, reduced affinity for $1,25(OH)_2D_3$, and disrupted RXR heterodimerization, to interference with coactivator interactions.[119,138] Increased understanding regarding the structure of the VDR and its interaction with ligand, RXR, and DNA will enable the development of small molecules that may circumvent disturbed VDR function. This will be discussed in more detail in Section 3.5 on treatment.

3.2 Clinical Picture

Children born with HVDRR develop hypocalcemia early in infancy, which often occurs within months after birth with clinical rickets ensuing soon thereafter.[118,119] Affected children suffer from bone pain, muscle weakness, hypotonia, and occasionally have convulsions due to the hypocalcemia. Pain in the lower extremities often delays their development of walking. Fractures and pseudofractures are common.[139–141] Affected children are usually growth retarded and in many cases the children develop severe dental caries or hypoplasia of the teeth.[117,119] The laboratory findings include low serum concentrations of calcium and phosphate and often strikingly elevated serum levels of alkaline phosphatase. The children exhibit secondary hyperparathyroidism with markedly elevated PTH levels. The serum 25(OH)D values are usually normal, except if concomitant vitamin D deficiency is also present. Serum $1,25(OH)_2D$ levels are almost always substantially elevated. This clinical finding distinguishes HVDRR from 1α-hydroxylase deficiency, in which case the serum $1,25(OH)_2D$ values are low or absent (Table 39.1). Many children with HVDRR have sparse body hair, while some have total scalp and body alopecia, including eyebrows and in some cases eyelashes (Fig. 39.3).[142] Alopecia, when present, also distinguishes HVDRR from 1α-hydroxylase deficiency.

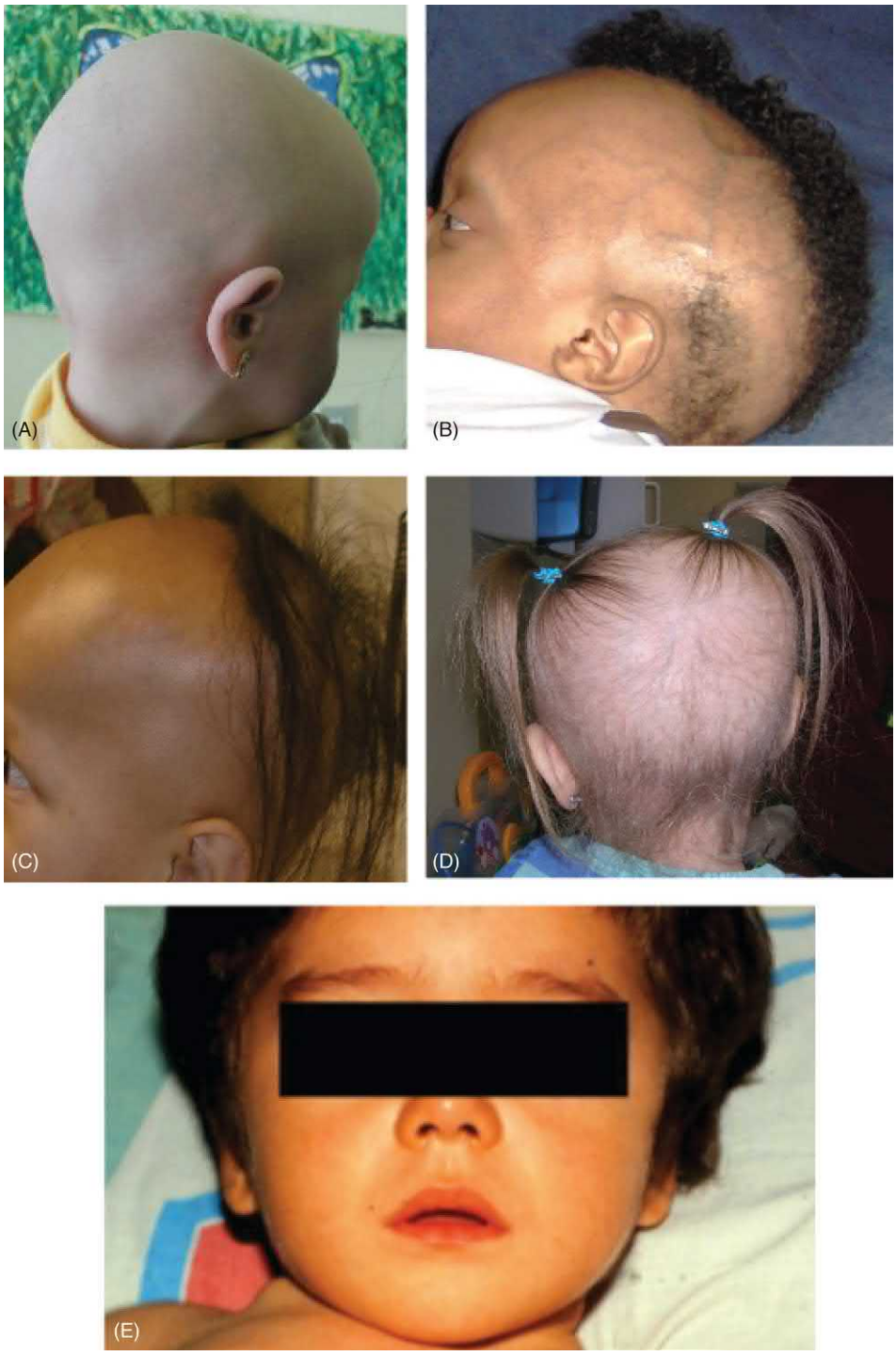

FIGURE 39.3 Variations in patterns of scalp hair from total alopecia to normal hair distribution in children with HVDRR. (A) Some children have total alopecia, (B) while others may have areas of normal hair adjacent to bald areas. (C–D) Children with the same mutation (R50X) may show different patterns of alopecia. (E) Children with severe hypocalcemia and rickets may not have alopecia depending on the nature of the defect in VDR. *Source: Figure used with permission from Malloy PJ, Feldman D. The role of vitamin D receptor mutations in the development of alopecia.* Mol Cell Endocrinol *2011;***347***:90–6.*[142]

Most affected children are resistant to therapy with supraphysiologic doses of any form of vitamin D, including calcitriol, which is an essential difference compared to the responsiveness of children with 1α-hydroxylase deficiency.

HVDRR is an autosomal recessive disease with equal prevalence in males and females. The parents of affected children, who are heterozygous carriers of the genetic trait, usually show no symptoms of the disease and have normal bone development. In most cases, consanguinity

is associated with the disease with each parent contributing a defective gene, although occasionally in nonconsanguineous cases, a different mutation has been found on each VDR allele (compound mutations) in the affected children.[143,144] In one recently published study, a child harboring a heterozygous VDR mutation (E420A) developed clinically severe HVDRR due to dominant negative suppression of the WT allele.[145] The father, who carried the heterozygous mutation, also had some bone disease when he was young and as an adult had modestly elevated $1,25(OH)_2D$ and PTH levels with slightly reduced serum calcium levels. The mother was entirely normal and carried no VDR mutation.

In the recent years, $1,25(OH)_2D$ signaling and single-nucleotide polymorphisms in the *VDR* gene have been associated with many extraskeletal clinical outcomes of vitamin D, such as asthma, autoimmune diseases, placental dysfunction, cancer risk and progression, cardiac hypertrophy, and energy metabolism.[146–157] Still, conclusive clinical data regarding the potentially beneficial effects of vitamin D on a number of disease processes are lacking.[156] Yet, most, if not all patients with HVDRR do not present with symptoms related to these end points, which may implicate that redundant mechanisms are in place to prevent potentially undesired consequences of HVDRR.[158]

3.3 Mutations in the VDR Gene That Lead to HVDRR

Over 100 cases of HVDRR have been recorded and a number of these have been analyzed at the biochemical and molecular levels.[117–120,159,160] Presently, 56 heterogeneous mutations have been identified in the *VDR* gene causative for HVDRR (Figs. 39.4 and 39.5; Tables 39.2 and 39.3). Mutations in the DBD prevent the VDR from binding to DNA, causing total resistance to $1,25(OH)_2D_3$ even though $1,25(OH)_2D_3$ binding to the VDR is

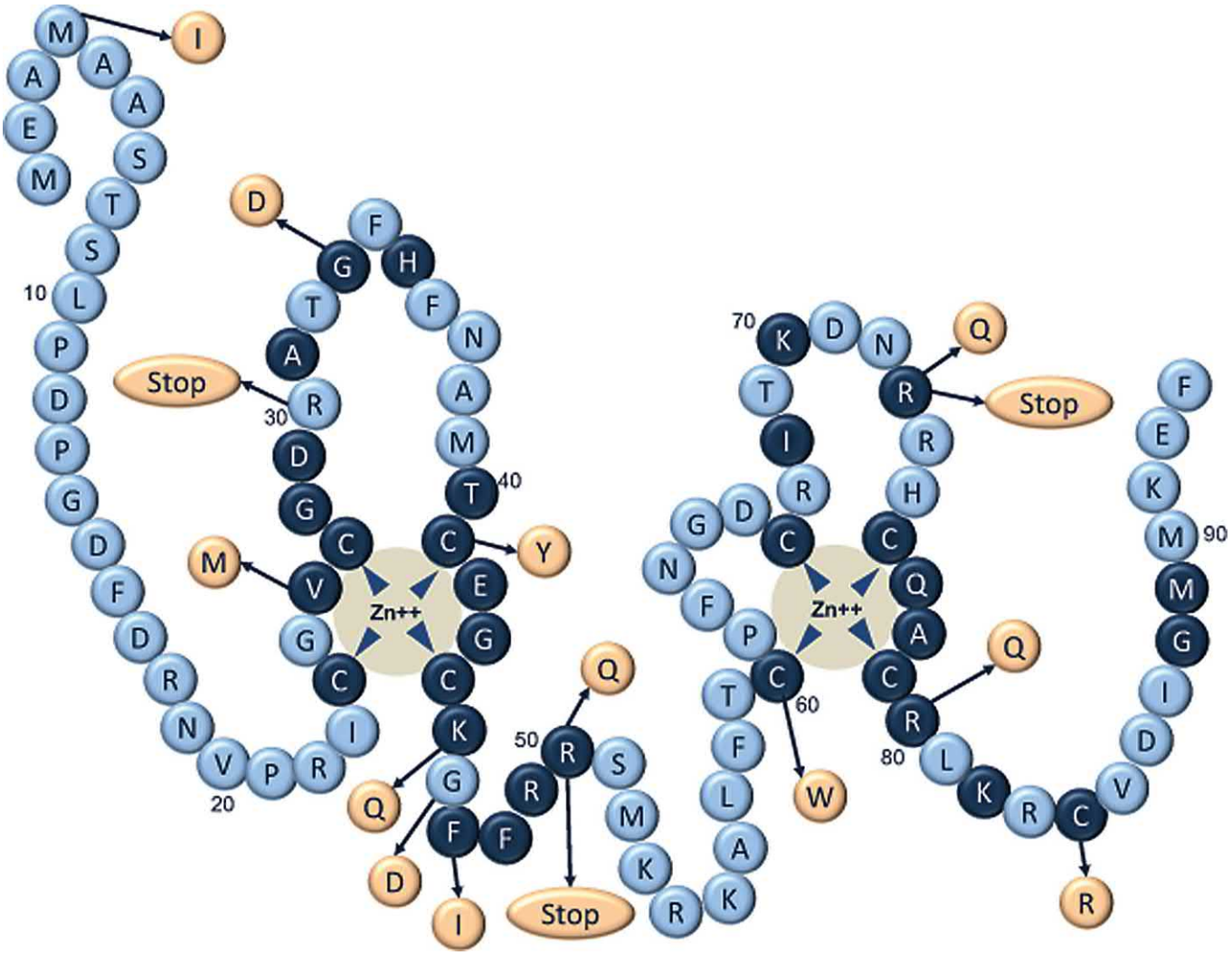

FIGURE 39.4 Missense mutations identified in patients with HVDRR. The locations of missense mutations are indicated in the VDR DBD which is depicted as a two–zinc finger structure.

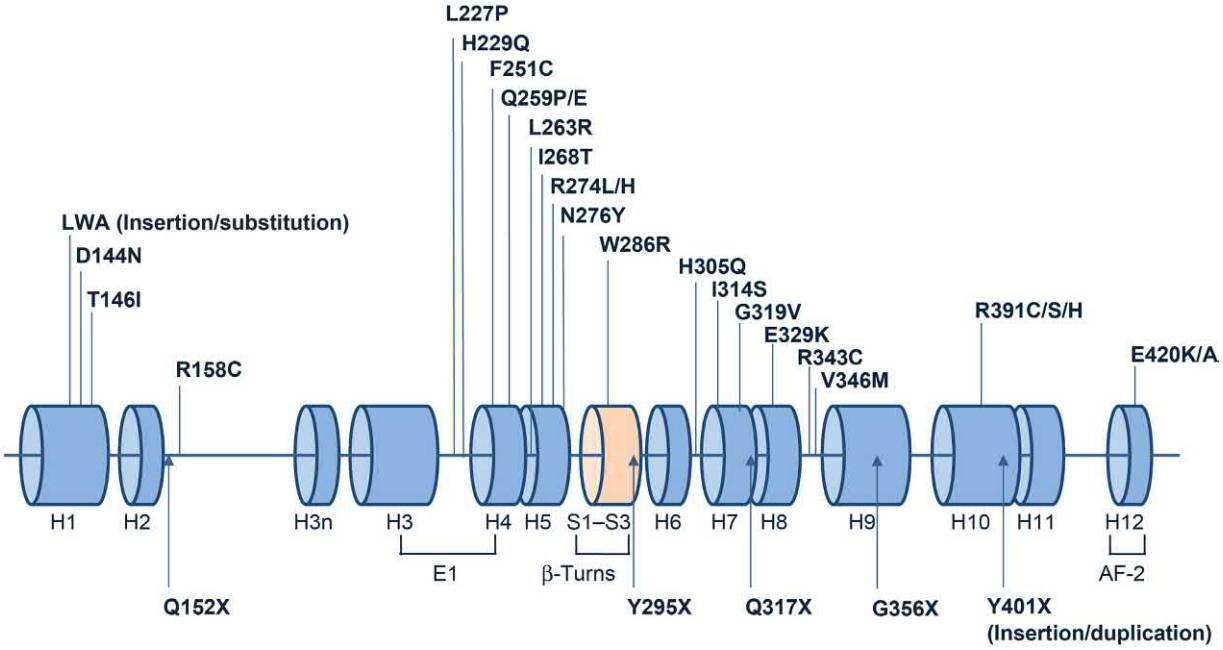

FIGURE 39.5 The locations of the missense mutations in the VDR LBD are shown above the *line*. The nonsense mutations are depicted below the *line*. The *filled disks* represent α-helices (*H1–H12*) and the disk depicting *S1–S3* represents a β-sheet structure. The locations of the *E1* and *AF-2* domains are also indicated.

unaffected. On the other hand, mutations in the LBD may disrupt ligand binding, heterodimerization with RXR, or prevent coactivators from binding to the VDR and cause partial or total hormone resistance. Other mutations that have been identified in the *VDR* gene causing $1,25(OH)_2D_3$ resistance include nonsense mutations (Figs. 39.4 and 39.5; Table 39.2), insertions/substitutions, insertions/duplications, deletions, and (donor/acceptor) splice site mutations (Table 39.3). Some of these VDR mutations will be described here.

We are aware of 13 missense mutations that have been identified in the VDR DBD as the cause of HVDRR (Fig. 39.4; Table 39.2). These mutant VDRs exhibit normal ligand binding but defective DNA binding.[117,119,120] A common feature of patients with mutations in the DBD is that they all have alopecia. The heterozygous parents with a single mutant allele are asymptomatic. In one case, a predicted "benign" variant (M4I) led to a pathogenic allele due to the presence of a *Fok*I polymorphism (M1T) in *cis* that disrupted the first two translational start sites.[161] However, the patient did not present with alopecia, despite reduced VDR transactivation activity, but with normal RXR heterodimerization.

Seven nonsense mutations have been identified in the *VDR* gene that truncate the VDR protein (Table 39.2).[117,119,120,160] Fibroblasts grown from skin biopsies taken from patients with *VDR* nonsense mutations in the LBD exhibit absent ligand binding and often the truncated VDR protein is not detected by immunoblotting. One particular mutation, a single base change in exon 8 that introduced a premature termination codon (Y295X), was identified in several families that comprise a large kindred where consanguineous marriages were common.[186,197] The *VDR* mRNA was undetectable by Northern blot analysis, indicating that the Y295X mutation led to nonsense-mediated mRNA decay. Interestingly, in one family with an R30X mutation, the parents of the affected child had somewhat elevated serum $1,25(OH)_2D$ levels, indicating some resistance to $1,25(OH)_2D$.[189] Tamura et al.[194] described a 2-year-old girl with an R73X mutation, which had been described previously in five different studies (Table 39.2). However, in this case HVDRR was caused by a process called uniparental duplication, causing inheritance of two copies from the maternal chromosome 12 allele carrying the *VDR* mutation instead of receiving one allele from both parents. All of the affected children with nonsense mutations had alopecia.

HVDRR can also be caused by splice site mutations in the *VDR* (Table 39.3). These mutations usually cause a frameshift and eventually introduce a downstream premature stop signal resulting in a nonfunctional VDR, such as the IVS3 + 4-5 mutation in the 5′-splice site of exon 3, where a 2-bp deletion leads to a premature stop.[160] Splice site mutations in the *VDR* gene cause exons to be skipped,[160,205,206] or cause incorporation of an intron into the *VDR* mRNA.[202] In one case, a cryptic 5′-donor splice site was generated in exon 6 that deleted 56 nucleotide bases and led to a frameshift in exon 7.[177] To date, only one mutation has been found in an acceptor splice site

TABLE 39.2 Missense and Nonsense Mutations in the VDR Gene Causing HVDRR

Types of mutations	Exons	Alopecia	References
Missense mutations in DBD			
M4I + *Fok*1 in *cis*	2	N	161
V26M	2	Y	138
G33D	2	Y	162,163
H35Q	2	Y	164
C41Y	2	Y	165
K45E	2	Y	166,167
G46D	2	Y	168
F47I	2	Y	167
R50Q	3	Y	169
C60W	3	Y	160
R73Q	3	Y	162
R80Q	3	Y	170,171
C84R	3	Y	172
Missense mutations in LBD			
D144N	4	N	160
T146I	4	N	173
R158C	5	N	160,173
L227P	6	N	174
H229Q	6	N	161
F251C	6	Y	175
Q259E	7	Y	176
Q259P	7	Y	177
L263R	7	Y	144
I268T	7	N	178
R274L	7	N	179
R274H	7	N	180
N276Y	7	N	160
W286R	8	N	181
H305Q	8	N	182
I314S	8	N	183
G319V	8	Y	176,184
E329K	8	Y	185
R343C	9	Y	186
V346M	9	Y	187
R391C	9	Y	183
R391H	9	Y	186
R391S	9	Y	144
E420K	9	N	188
E420A (heterozygous mutation)	9	N	145

TABLE 39.2 Missense and Nonsense Mutations in the VDR Gene Causing HVDRR (*cont.*)

Types of mutations	Exons	Alopecia	References
Nonsense mutations in DBD			
R30X	2	Y	143,184,186,189,190
R50X	3	Y	191,192
R73X	3	Y	176,177,193–195
Nonsense mutations in LBD			
Q152X	4	Y	179,196
Y295X	7	Y	186,197,198
Q317X	8	Y	199
G356X	9	Y	200

TABLE 39.3 Other Types of Mutations in the VDR Gene Leading to HVDRR

Types of mutations	Exons	Alopecia	References
Deletions			
delAG (IVS3Δ + 4-5)	Exon 3–intron E	Y	160
c.366delC (c.526delC)	4	Y	185
c.716delA (c.879delA)	6	Y	201
ΔK246 (c.898delCTT)	6	Y	143
Deletions/substitutions			
5-bp deletion/8-bp substitution	4	N	202
Insertions/duplications			
102-bp insertion/duplication	9	Patchy	203
Splice site mutations			
IVS2-2ANT	Intron 2	Y	204
IVS3 + 5GNC	Exon 3–intron E	Y	193,205
GTCAGT to GTGAGT (c.862CNG)	6	Y	177
IVS4 + 1GNC	Exon 4–intron F	Patchy	202
IVS8 + 1GNT	Exon 8–intron J	Patchy	206

(c.147 – 2A>T), but the consequences of this intronic mutation on *VDR* expression are not known.[204]

Currently we are aware of 25 missense mutations that have been identified in the VDR LBD (Fig. 39.5; Table 39.2).[119,120,183,207] All these mutations lead to reduced or absent binding of $1,25(OH)_2D_3$ and/or also affect heterodimerization with RXR. One patient had an R274L mutation that altered the contact point for the 1α-hydroxyl group of $1,25(OH)_2D_3$, lowering the binding affinity of the VDR for $1,25(OH)_2D_3$.[135,207] A second patient had an H305Q mutation that altered the contact point for the 25-hydroxyl group of $1,25(OH)_2D_3$.[135,182] This mutation also lowered the binding affinity of the VDR for $1,25(OH)_2D_3$. These cases illustrate the importance of critical amino acids as contact points for $1,25(OH)_2D_3$ and demonstrate that mutations of these residues can often be the basis for HVDRR.

The R391C mutation in the VDR LBD had no effect on ligand binding, but disrupted heterodimerization with RXR. R391 is located in helix H10 where the RXR dimerization interface is formed from helix H9 and helix H10 and the interhelical loops between H7–H8 and H8–H9 in VDR.[135] In two other cases, R391 was mutated to a serine (R391S) or histidine (R391H) residue.[144,186] Several other

mutations have been identified in the VDR that affect VDR:RXR heterodimerization, including G319V, Q259P, Q259E, and F251C.[175–177,184] A V346M and a nearby R343C mutation were identified in patients with HVDRR that may be important in RXR heterodimerization.[186,187] All of the patients with defects in VDR:RXR heterodimerization had alopecia.

A very informative mutation has been identified in the VDR that prevents coactivator recruitment, which is critical for transcriptional activity.[188] An E420K mutation located in helix H12 caused no defect in multiple steps in VDR gene regulation, including ligand binding, VDR:RXR heterodimerization, and DNA binding. However, the E420K mutation abolished VDR binding by the coactivators SRC-1 and DRIP205. Interestingly, the child with the E420K mutation did not have alopecia, although full-blown rickets was present.[188] Importantly, this finding suggested that ligand-mediated transactivation and coactivator recruitment by the VDR, which is crucial to prevent rickets, is not required for normal hair growth.

Thus far, five compound heterozygous mutations in the VDR gene have been identified in children with HVDRR. In these cases each heterozygous parent harbored a different mutant *VDR* gene and parental consanguinity was not involved. One patient was heterozygous for an E329K mutation and had a second mutation on the other *VDR* allele that deleted a single nucleotide 366 (366delC) in exon 4.[185] The single-base deletion resulted in a frameshift, creating a premature termination signal that truncated most of the LBD. The E329K mutation in helix H8, which is important in heterodimerization with RXR, likely disrupted this activity. In a child with HVDRR and early childhood–onset type 1 diabetes, compound heterozygosity for L263R and R391S mutations in the *VDR* gene was found.[144] The mutant VDRs in this case exhibited differential effects on 24-hydroxylase and RelB promoters. The 24-hydroxylase response was abolished in the L263R mutant, but only partially affected in the R391S mutant. On the other hand, RelB responses were normal for the L263R mutant, but the R391S mutant failed to induce RelB promoter activity.[144] The reason for the differential activities of these VDR mutants is unknown. Zhou et al.[143] described a patient with HVDRR and alopecia harboring a heterozygous nonsense mutation R30X and a 3-bp deletion in exon 6 that deleted the codon for lysine at amino acid 246 (ΔK246). The ΔK246 mutation did not affect ligand binding but abolished heterodimerization with RXR and binding to coactivators.[143]

In contrast to these three patients with compound heterozygous mutations in the *VDR* and having alopecia, a compound heterozygous HVDRR patient was recently described that did not present with alopecia.[161] As mentioned earlier, the patient had a heterozygous M4I mutation, which was harmless on its own but turned out to be pathogenic owing to an additional *Fok*I polymorphism. Besides, the patient harbored a H229Q mutation, which reduced VDR-activated transcription but VDR:RXR heterodimerization remained intact.[161] The H229Q mutation is located in a part of the LBD that does not lead to alopecia, such as L227P,[174] but the M4I/*Fok*I mutation unexpectedly also does not, resulting in a patient with normal scalp hair.[161] A compound heterozygous patient was described by Song et al.[173] in which two mutations were discovered within the LBD, T146I in exon 4 and R158C in exon 5. As expected, all signs of HVDRR were observed, except alopecia.

One insertion/duplication in the *VDR* gene causing HVDRR has been reported.[208] A unique 5-bp deletion/8-bp substitution led to deletion of amino acids H141 and Y142, which were substituted with three amino acids (L141, W142, and A143). In experiments to unravel the cause of the defect, only the A143 insertion into the WT *VDR* disrupted transactivation to the same extent as the natural mutation. The patient with this mutation did not have alopecia. A unique 102-bp insertion/duplication was found in the VDR gene that introduced a premature stop (Y401X) and resulted in deletion of helix H12.[203] The truncated VDR was able to heterodimerize with RXR, bind to DNA, and interact with the corepressor hairless (HR) but failed to bind coactivators and was transactivation defective. The patient with this mutation had patchy alopecia.

There are two reported cases where investigators failed to detect a mutation in the *VDR* as the basis of HVDRR.[186,209] Whereas in the former[186] no explanation was provided, in the latter[209] the authors speculated that the resistance to the action of $1,25(OH)_2D_3$ was due to abnormal expression of hormone response element–binding proteins belonging to the hnRNP family that prevented the VDR:RXR complex from binding to vitamin D response elements in target genes.[210]

3.4 Animal Models

Mouse models of HVDRR have been created in the laboratory by several groups using targeted ablation of the VDR, where the targeted region has been the DBD domain.[146,211–213] The *VDR*-null mice (VDRKO) recapitulate the findings in the children with HVDRR. VDRKO mice appear normal at birth and become hypocalcemic with slightly elevated PTH levels after weaning. Bone mineralization is severely impaired and the rachitic changes progress over time. VDRKO mice have normal hair at birth but develop progressive alopecia, thickened skin, enlarged sebaceous glands, and epidermal cysts.[211,212]

In one VDRKO mouse model uterine hypoplasia with impaired folliculogenesis was found in female reproductive organs, but it was not completely clear if this was secondary to hypocalcemia or to the VDR defect.[212] Moreover,

specific effects have been described for VDR dysfunction in testis and placenta.[152,214] Importantly, VDR haploinsufficiency revealed temporal sex-specific effects on bone and energy metabolism.[215] Moreover, global VDRKO mice develop sex-related differences in their skeleton during aging, emphasizing the urge to assess both sexes when studying VDR function in mouse models.[216]

When VDRKO mice are fed a "rescue" diet, their calcium can be normalized and the rickets reversed or prevented, as was described for children with HVDRR successfully treated with intravenous (IV) or oral calcium. Many nonskeletal abnormalities seen in the hypocalcemic mice are prevented by the rescue diet, indicating that the abnormalities resulted from the hypocalcemia and were not directly caused by the absence of a functional VDR. Nevertheless, as in children with HVDRR, alopecia is not reversed or prevented by normalization of calcium homeostasis.

Recent genetic developments, employing conditional tissue- or cell-specific VDRKO mice has increased our knowledge on the specific role of VDR in a given tissue or cell type.[146,156,217,218] Despite disturbed dietary calcium uptake, mice lacking VDR in intestinal cells have normal calcium levels, which derives from the mobilization of skeletal calcium.[219] In fact, perturbed VDR signaling in the intestine increases vitamin D signaling–mediated enhanced bone resorption and reduces mineralization of bone. Still, the bone phenotype of various osteoblast- and osteocyte-specific VDRKO and transgenic mice has not been fully clarified and seems to depend on the differentiation stage of bone cells.[219–222] Of interest, mice lacking VDR in macrophages develop undesired metabolic phenotypes, including insulin resistance and atherosclerosis,[154] further implicating a role for vitamin D in metabolism.[223] These and future studies in conditional mice will allow us to study the direct role of VDR in a detailed spatial and temporal manner.

A spontaneous case of HVDRR in a Pomeranian dog has been described.[224] The dog had characteristic chemistries of HVDRR with marked hypocalcemia, elevated $1,25(OH)_2D$ levels, and partial alopecia. The *VDR* mutation was a deletion causing a frame shift at R175 and a premature termination of the protein. The dog had severe bone disease and developed a spinal fracture of T11 causing acute spinal cord compression requiring euthanization.

3.5 Treatment

Mutations in the VDR that cause HVDRR result in partial or total resistance to $1,25(OH)_2D_3$ action.[118–120] Despite elevated levels of $1,25(OH)_2D_3$, the patients become hypocalcemic, predominantly due to a lack of VDR signaling in the intestine to promote dietary calcium absorption. The hypocalcemia leads to a decrease in bone mineralization and causes rickets. Some HVDRR patients improve both clinically and radiologically when treated with pharmacological doses of vitamin D ranging from 5,000 to 40,000 IU/day, 20–200 μg/day of $25(OH)D_3$, and 17–20 μg/day of $1,25(OH)_2D_3$.[117,225] Some patients also responded to $1\alpha(OH)D_3$.[117,119] The patient with the H305Q mutation, a contact point for the 25-hydroxyl group of $1,25(OH)_2D_3$, showed improvement with 12.5 μg/day calcitriol treatment.[182,226] On the other hand, the patient with the R274L mutation, a contact point for the 1α-hydroxyl group of $1,25(OH)_2D_3$, was unresponsive to treatment with 600,000 IU vitamin D, up to 24 μg/day of $1,25(OH)_2D_3$, or 12 μg/day $1\alpha(OH)D_3$.[227]

When patients fail to respond to vitamin D or $1,25(OH)_2D_3$, which is often the case, intensive calcium therapy is used.[118,142] Oral calcium can be absorbed in the intestine by both vitamin D–dependent (predominantly transcellular) and vitamin D–independent (predominantly paracellular) pathways. In children with nonfunctional VDR, the vitamin D–independent pathway is critical. When oral calcium therapy is successful, the calcium levels in the gut have been raised high enough for passive diffusion or other nonvitamin D–dependent absorption to establish and maintain normocalcemia.

IV calcium infusion treatments are the last resort for children with HVDRR who failed prior treatments with large doses of vitamin D derivatives and/or oral calcium.[228–232] Delivery of calcium by the IV route circumvents the calcium absorption defect in the intestine caused by the defective VDR.[119,138,191] However, in affected children receiving IV calcium, the syndrome recurs slowly over time upon discontinuation of the IV therapy. Some children have been managed with intermittent IV calcium regimens using oral calcium in the intervals.[191,206] Once the child becomes older, perhaps due to the skeleton ceasing longitudinal growth, oral calcium often suffices to maintain normocalcemia and the IV calcium regimen can be discontinued.[119,232] In some cases, oral calcium alone has been successfully used as a first-line therapy for HVDRR patients.[119,166,233] To our knowledge, only one case has been reported where a gastric tube had to be used to adequately restore calcium homeostasis by 24 h continuous enteral infusion of calcium chloride.[174] This was done because the patient developed recurrent IV line sepsis and several other enteral preparations turned out to be ineffective.

Chaturvedi et al.[195] and Fudge and Kovacs[234] reasoned that during fetal development, VDR-independent pathways are operative in the placenta, which may explain the apparently normal mineral metabolism in infants with HVDRR. Intriguingly, spontaneous healing of rickets into adulthood has been observed in some HVDRR patients as they grow up and all therapy sometimes can

be discontinued.[119,166,184,235–237] Nevertheless, if alopecia is present in these patients, it cannot be undone by the treatment despite normalization of calcium and healing of rickets.[142] According to Tiosano et al.[238] and in line with the VDR-independent actions in the placenta, a time window may exist between the age of 18 and 30 years that allows enhanced fractional calcium absorption in the intestine, most likely paracellular and independent of VDR function, and that may explain the normalized bone phenotype despite no medication intake. This limited time window also suggests that calcium homeostasis in patients with HVDRR should be monitored closely later in life.

An early unexpected finding was that raising the serum calcium to normal by IV or oral calcium administration reversed all aspects of HVDRR, including hypocalcemia, hypophosphatemia, secondary hyperparathyroidism, rickets, elevated alkaline phosphatase, etc., but not alopecia.[117] It is worthwhile mentioning that for a patient with alopecia areata and reduced VDR expression, although not diagnosed with HVDRR, recovery of alopecia areata was achieved by topical application of a strong vitamin D analog, calcipotriol.[239]

Correcting the hypocalcemia often corrects the hypophosphatemia, as well as suppresses the PTH without the need for phosphate supplements, although some children with extremely decreased or difficult-to-normalize serum phosphate have been treated with phosphate.[174,206] This finding indicates that the low phosphate is caused by the secondary hyperparathyroidism, which normalizes with correction of the hypocalcemia, even in the absence of VDR action. The inescapable conclusion is that the most important actions of $1,25(OH)_2D_3$ on calcium and bone homeostasis take place in the intestine through calcium absorption and not in the skeleton. The ability of the rachitic bone abnormality to normalize in the absence of VDR-mediated vitamin D action has been surprising. In patients, data are incomplete pertaining to the complete normalization of microstructure and quality of the skeleton. Careful examination of the bone histology in VDRKO mice suggests that subtle defects remain in the bones or when aging ensues in *VDR*-null mice whose serum calcium had been corrected by a rescue diet.[216,240,241] However, the reversal of all clinical aspects of HVDRR with IV calcium does indicate that healing of bone and reversal of secondary hyperparathyroidism and hypophosphatemia can take place without normal VDR-mediated vitamin D action.[119] There is no doubt that vitamin D has important actions on bone and parathyroid cells.[242] However, these actions can apparently be compensated for in vivo if the calcium level is normalized.

In an attempt to develop novel treatment modalities to treat HVDRR patients, many studies have been devoted to generate VDR analogs or small molecules that have a higher affinity for the VDR compared to $1,25(OH)_2D_3$ or might bind and act upon mutant VDR.[243,244] For example, calcipotriol and doxercalciferol have been approved for the treatment of psoriasis, osteoporosis, or secondary hyperparathyroidism.[245] Besides screening for analogs in cell and animal models, several studies report on the deliberate introduction of mutations in the *VDR* to assess the role of specific residues or regions of the VDR in ligand binding, heterodimerization, or DNA binding.[126,127,129,246]

Lithocolic acid (LCA), a bile acid, has been reported to bind and activate the VDR.[247–249] The importance of this activation step to VDR actions, especially in the gut, is still not completely understood. Curcumin, a turmeric-derived bioactive polyphenol, can also bind the VDR and potentially have actions in humans.[250]

It was reported that an engineered mutation at position 305 of the *VDR* was detrimental for transactivation by $1,25(OH)_2D_3$, but it increased sensitivity for LCA.[126] A similar finding was reported for a C410Y mutation, which also enhanced LCA sensitivity.[127] A mouse model was created carrying a point mutation in the *Vdr* (L304H), rendering $1,25(OH)_2D_3$ binding impaired but acquiring sensitivity for so-called vitamin D "gemini" analogs with double side chains.[129] Indeed, gemini analogs were able to prevent the three major consequences of unliganded VDR in this model, that is, disturbed calcium homeostasis, bone loss, and alopecia, of which only the latter could also be prevented with $1,25(OH)_2D_3$. Lee et al.[251] discovered that removal of small uniquitin-like modifier (SUMO) from its acceptor site at position 91 of the VDR DBD by sentrin/SUMO proteases (SENPs) potentiates transactivation of the VDR by $1,25(OH)_2D_3$ and heterodimerization with the RXR. On the other hand, removal of endogenous sentrin/SUMO proteases abolishes *CYP24* expression, which further emphasizes the important regulatory role of SUMOylation in the signaling activity of the VDR. These studies combined suggest that, depending on the location or nature of the mutation, HVDRR patients may actually benefit from therapy with vitamin D precursors or analogs, rather than $1,25(OH)_2D_3$ itself. Needless to say, this area of research requires much more study before analogs will surface for treatment of HVDRR patients.

In recent years there have been many new actions attributed to vitamin D that are thought, but not yet proven in human trials, to mediate important and wide-spread effects that are unrelated to calcium and bone homeostasis.[146,156,242] As alluded to in Section 3.2, these include actions to reduce the risk of cancer, autoimmune disease, infection, neurodegeneration, hypertension, and cardiac disease. At this time we have not detected a trend toward an increased risk for any of these potential problems in children with HVDRR.[145] Perhaps, redundant

mechanisms are in place to prevent potentially undesired consequences of HVDRR.[158] On the other hand, there are very few cases of HVDRR and most of them are detected in infants and young children, thus precluding the detection of an increased tendency toward any of these potential health problems that usually occur in older individuals.

3.6 Alopecia

The molecular analysis of the VDR from HVDRR patients with and without alopecia has provided several clues to the functions of the VDR that are important for normal hair growth.[142] For example, children with vitamin D deficiency or with 1α-hydroxylase deficiency do not develop alopecia, suggesting that absence of vitamin D action is not the cause of alopecia. Patients with premature stop mutations and VDRKO mice have alopecia, suggesting that the intact VDR protein may be critical for renewed hair growth after birth.[211,212] Expression of the WT VDR in keratinocytes of VDRKO mice prevented alopecia, a finding that further supports a role for the VDR itself in regulating hair growth and a critical role for keratinocytes.[252] Patients with DBD mutations also have alopecia, indicating that VDR binding to DNA is critical to prevent alopecia. As described in Section 3.3, one exception has been reported of a patient with compound heterozygosity for a mutation not only in the DBD (M4I/*Fok*I polymorphism), but also in the LBD (H229Q) that, based on nearby known mutations, probably would not have led to alopecia if it concerned a homozygous mutation.[161] In addition to mutations in the DBD, patients with *VDR* mutations that inhibit RXR heterodimerization have alopecia, indicating an essential role for VDR:RXR heterodimers in hair growth.[175,177,183] In line with this, inactivation of RXRα in keratinocytes of mice also caused alopecia.[253] In contrast to the patients with mutations in the DBD or that inhibit RXR heterodimerization, patients with *VDR* mutations that abolish ligand binding do not have alopecia. This suggests that a ligand-independent action of the VDR may regulate the normal hair cycle.[142,146,188,254–256] The patient with the E420K mutation, which abolished coactivator binding (but not ligand binding or RXR heterodimerization), did not have alopecia despite suffering from severe rickets, implicating that VDR actions to regulate hair growth were independent of coactivator interactions.[188] Also, when ligand- or coactivator-binding defective mutant VDRs were specifically expressed in keratinocytes in VDRKO mice that have alopecia, hair growth was fully or partially restored.[256] Cumulatively, the data suggest that unliganded VDR is an essential factor that prevents alopecia.

As shown in Fig. 39.3, various patterns of alopecia occur, ranging from total absence of hair to diffuse sparse hair, to areas of absent hair adjacent to areas with hair. No correlation seems to exist between the alopecia pattern and the type of mutation present. In fact, unrelated children with the same mutation showed different patterns of alopecia (Fig. 39.3).[191] The mechanism behind the development of these diverse patterns of alopecia is currently unknown.

Regarding the mechanism by which unliganded VDR prevents alopecia, the definitive answer is still unclear. The alopecia associated with HVDRR is clinically and pathologically indistinguishable from the generalized disease atrichia with papular lesions found in patients with mutations in the *HR* gene.[185,257,258] The *HR* gene product HR acts as a VDR corepressor, directly interacts with the VDR, and suppresses $1,25(OH)_2D_3$-mediated transactivation.[254,258–260] It has been hypothesized that the role of the VDR in the hair cycle is to repress the expression of a gene(s) in a ligand-independent manner.[146,188,254,256,258] The ligand-independent activity requires that the VDR heterodimerize with RXR and bind to DNA.[188,255] The corepressor actions of HR may also be required for the unliganded VDR to repress gene transcription during the hair cycle. Mutations in the *VDR* that disrupt the ability of the unliganded VDR to suppress gene transcription are hypothesized to lead to the derepression of a gene(s) whose product, when expressed inappropriately, disrupts the hair cycle that ultimately leads to alopecia.[146,188,254,256,258] Inhibitors of the Wnt signaling pathway are possible candidates.[142,261–263] Thus far, there have been no reports of mutations in the VDR that affect interactions with HR. The role of HR in regulating the unliganded action of the VDR during the hair cycle remains to be discovered. Recently, a role for the VDR coactivator mediator 1 (MED1) has been suggested in the development of hair loss.[264] Using epidermal-specific *Med1*-deficient mice, hair loss was observed similar to that in *Vdr*- or *Hr*-deficient mice,[265,266] but other findings suggested VDR coactivator–independent roles of MED1 in hair loss. This study indicates that novel associated genes in the process of hair growth are yet to be found, raising opportunities to find novel targets for the treatment of HVDRR patients with alopecia. More recently, Demay and coworkers[267] showed by chromatin immunoprecipitation analyses that the unliganded VDR was recruited to the regulatory regions of cWnt and hedgehog target genes in WT keratinocytes but not in $VDR^{-/-}$ keratinocytes. Their data revealed that the unliganded VDR interacts with regulatory regions in the cWnt and hedgehog target genes and is required for the induction of pathways regulated by cWnt and hedgehog during the postnatal hair cycle.[267] These data solidify the hypothesis that unliganded VDR is required for the postnatal development of hair and appear to explain the alopecia in HVDRR.

4 CONCLUSIONS

The biochemical and genetic analyses of CYP27B1 and CYP2R1 in 1α- and 25-hydroxylase–deficient patients, respectively, as well as the VDR in HVDRR patients, have yielded important insights into the structures and functions of the critical enzymes in hormone synthesis and the receptor that mediates $1,25(OH)_2D$ action. Similarly, studies of the affected children with 1α-hydroxylase deficiency and HVDRR continue to provide a more complete understanding of the biological role of $1,25(OH)_2D$ in vivo. A concerted investigative approach to 1α-hydroxylase deficiency and HVDRR at the clinical, cellular, and molecular levels has proven exceedingly valuable in gaining knowledge about the functions of the different domains of the 1α-hydroxylase and the VDR proteins and in elucidating the detailed regulation of the synthesis and mechanism of action of $1,25(OH)_2D$. Although challenges remain, these studies have been essential to promote the well-being of the families with 1α-hydroxylase deficiency and HVDRR and in improving the diagnostic and clinical management of these rare genetic diseases.

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CHAPTER

40

Heritable Renal Phosphate Wasting Disorders

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1 INTRODUCTION

Phosphorus is one of the most abundant constituents of the body and disturbances in phosphate homeostasis can affect almost any organ system.[1] Deficiency of this mineral is capable of profoundly compromising a variety of tissues. Depending on its rapidity of onset and severity, hypophosphatemia can lead to rickets, osteomalacia, cardiomyopathy, rhabdomyolysis, metabolic acidosis, and red cell dysfunction. As inorganic phosphate (Pi) homeostasis and vitamin D metabolism are reciprocally regulated,[2] it is often difficult to know whether the consequences of hypophosphatemia are directly related to changes in $1,25(OH)_2D$ production. Discrimination between the possibilities has frequently been achieved by evaluating the response of hypophosphatemic patients to Pi supplementation. Few among the hypophosphatemic disorders respond adequately to Pi supplementation alone, whereas coincident modification of vitamin D status can promote disease regression. Herein, we provide an overview of important concepts related to Pi homeostasis and the regulation of vitamin D metabolism necessary to understand, diagnose, treat, and investigate the heritable disorders that feature hypophosphatemia from renal Pi wasting discussed later in this chapter.

2 PHOSPHATE HOMEOSTASIS

The majority of human phosphate (85%) is found in bone where it associates with calcium. Phosphate in blood and other extracellular fluids comprises only 1% of body phosphate, yet under most conditions serum Pi concentration correlates with the total body phosphate content. The kidney is the major regulator of extracellular Pi levels, and thereby plays a central role in facilitating skeletal mineralization and growth. Pi is filtered at the glomerulus and is reabsorbed primarily in the proximal tubule; ~60% in the proximal convoluted tubule and 15%–20% in the proximal straight tubule.[3] A remaining small, but variable, portion (<10%) of filtered Pi is reclaimed in more distal segments of the nephron. The amount of Pi reabsorbed by the proximal tubule is hormonally regulated, and is a key determinant of the serum Pi concentration in health or with a moderately reduced glomerular filtration rate (GFR).[4,5]

Renal Pi transport is effectively unidirectional, involving at first uptake by the renal tubule cell at its brush border membrane that is in contact with the urine, and then efflux at the basolateral membrane into the circulation.[6] Apical sodium-dependent Pi (Na^+/Pi) cotransport across the luminal (brush border) membrane is rate limiting and the target for physiological/pathophysiological alterations. At the apical domain of renal proximal tubular cells, two type 2 Na^+/Pi cotransporters, NPT2a *(SLC34A1)* and NPT2c *(SLC34A3)*, are expressed and modulate renal Pi transport that depends on Na^+, K^+-ATPase to maintain the Na^+ gradient that drives the transport system.[7] In contrast, the basolateral Pi transport system is not well defined, but efflux of Pi across the basolateral membrane may involve: (1) an anion exchange mechanism and/or a "Pi leak" to complete transcellular reabsorptive flux, and (2) a Na^+-dependent Pi uptake mechanism to guarantee Pi availability from the

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interstitium if apical influx is insufficient to maintain cellular metabolism.

The NPT2a cotransporter is located almost exclusively in the apical membrane of renal proximal tubular cells.[8] In mice, targeted disruption of the *Npt2a* gene, resulting in loss of 70%–80% of brush border membrane Na^+/Pi cotransport, reveals the crucial role for the type 2a receptor. This relationship is supported by the reported correlation of NPT2a protein abundance in brush border membranes with Na^+/Pi cotransport activity under a variety of physiological/pathophysiological conditions.[8] In these knock-out mice, there is also impaired urinary excretion of the inhibitor of mineralization, osteopontin.[9] In humans, loss-of-function mutations in the *NPT2a* gene, or hormonally-driven downregulation of the NPT2a protein, causes increased urinary Pi excretion, hypophosphatemia, and nephrocalcinosis or nephrolithiasis.[5]

The NPT2c cotransporter too is located at the apical domain of renal proximal tubular cells, and when mutated in humans results in hypophosphatemia, rickets, hypercalciuria, and kidney calcification designated hereditary hypophosphatemic rickets with hypercalciuria (HHRH) (OMIM #241530).[10] In contrast, mice null for *Npt2c* exhibit no renal Pi wasting when fed a normal phosphorus diet, suggesting that NPT2a is actually the major regulator of renal Pi transport. However, Segawa et al.[11] demonstrated that mice null for both *Npt2a* and *Npt2c* had more severe Pi wasting and rickets than mice null for *Npt2a* or *Npt2c* alone, indicating a synergism for murine Pi balance.

Two other Na^+/Pi cotransporters, type 1 and type 3, are expressed in the kidney. NPT1 *(SLC17A1)* is found at the apical membrane of proximal tubular cells and in the liver. It is a nonspecific anionic carrier whose physiological role regarding Pi homeostasis is still unknown.[8,12] The type 3 Pi transporter family is composed of PiT1 *(SLC20A1)* and PiT2 *(SLC20A2)*. These proteins, initially identified as retrovirus receptors, transport Pi with high affinity and are detected in all nephron segments.[7] Thus, the type 3 Na^+/Pi cotransporters may control basolateral Pi influx in all tubule cells to maintain cell metabolism, as well as in proximal tubule cells under conditions of limited apical Pi influx.

Proximal tubular Na^+/Pi cotransport is regulated by a variety of hormones/metabolic factors that alter NPT2a and NPT2c protein availability. Parathyroid hormone (PTH)-dependent inhibition of Na^+/Pi cotransport is initiated when PTH binds to the type 1 PTH receptor (PTH1R) in proximal tubular cells and stimulates intracellular second messengers that internalize the cell-surface NPT2a protein.[13] Control of this internalization requires the presence of Na^+-H^+ exchanger regulatory factor 1 (NHERF-1), which is a scaffolding protein that belongs to the PDZ domain protein family and contains two PDZ regions that bind the C-terminal end of NPT2a and PTH1R (Fig. 40.1). Targeted disruption of murine *Nherf1* results in a phenotype resembling *Npt2a*$^{-/-}$ mice, explained instead by aberrant localization of the NPT2a transporter within the proximal tubule cells and the consequent paucity of NPT2a at the renal brush border membrane.[14]

Fibroblast growth factor 23 (FGF23) inhibits renal Pi reabsorption by decreasing expression of NPT2a and

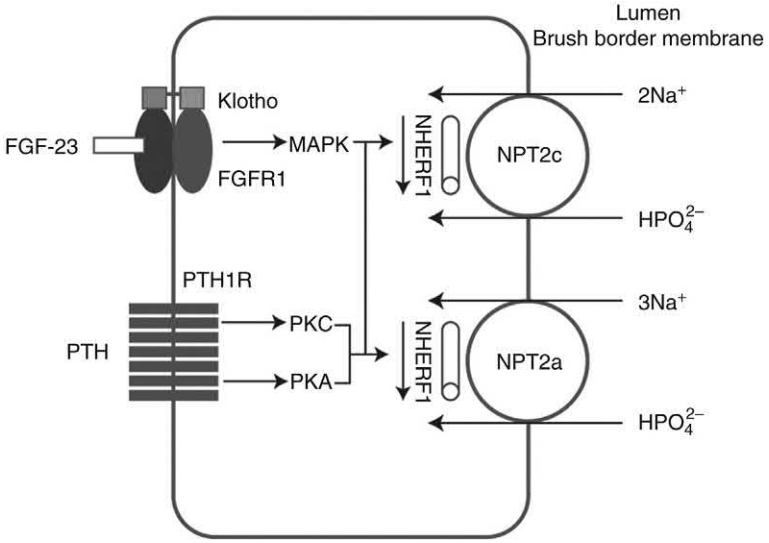

FIGURE 40.1 A summary of the biochemical pathways for parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF-23) mediated inhibition of Pi transport in the renal proximal convoluted tubule. PTH interacts with the PTH1 receptor of cells to activate PKC and PKA, resulting in the phosphorylation of Na^+-H^+ exchanger regulatory factor 1 (NHERF-1) and the dissociation of NPT2a from NHERF-1. The unbound NPT2a transporter then interacts with other proteins that facilitate its endocytosis (internalization) from the apical membrane, resulting in decreased Pi transport. FGF-23 binds to the FGF1 receptor requiring the membrane-bound protein klotho. Interaction of the heteromeric FGF1-klotho receptor elicits activation of MAPK and subsequent phosphorylation of NHERF-1, resulting in internalization of NPT2a and NPT2c.

NPT2c (Fig. 40.1). FGF23 does not regulate fetal Pi metabolism, but begins its effect 12 h postnatally,[15] yet is not associated with the physiological age-related changes in circulating levels or urinary handling of Pi across childhood.[16] FGF23 binds to the renal FGFR1 receptor and the membrane-bound protein, klotho, located primarily in the distal tubule. There it activates the heteromeric FGFR1/klotho receptor to elicit mitogen-activated protein kinase (MAPK) signaling in the distal convoluted tubule. This downregulates NPT2a and NPT2c expression in the proximal convoluted tubule by a paracrine process that involves phosphorylation of extracellular receptor kinase 1/2 (ERK1/2) and FGFR1 substrate[17–19] and most importantly phosphorylation of NHERF-1, which internalizes NPT2 cotransporters.[20] The crucial role for klotho protein has been confirmed using klotho-deficient mice. For them, FGF23 is ineffective in controlling serum Pi levels.[19]

Acute renal adaptation to Pi restriction is associated with an increase in renal proximal tubule cell brush border membrane NPT2a protein content. This is rapidly reversed by a high Pi diet.[21] In fact, NPT2a protein availability modulates a large number of Pi homeostatic disorders. However, changes in NPT2a protein expression and Na^+/Pi cotransport occur predominantly with constant amounts of *NPT2a* mRNA. NHERF-1 seems to help maintain steady-state NPT2a levels.[22] Rapid increments or decrements in Na^+/Pi cotransport follow, respectively, membrane insertion of NPT2a protein, which may be preceded by its de novo synthesis, or membrane retrieval of NPT2a protein followed by lysosomal degradation.[8] Although such membrane trafficking of NPT2a is central to the regulation of Na^+/Pi cotransport, changes in *NPT2a* mRNA levels do occur following chronic alterations in dietary Pi, prolonged treatment with $1,25(OH)_2D$, PTH, FGF23, or thyroid hormone, and in disorders of Pi homeostasis, such as X-linked hypophosphatemia (XLH) (see later). Thus, under select conditions, a transcriptional mechanism too regulates Na^+/Pi cotransport.

The intracellular mechanisms that direct insertion and retrieval of NPT2a protein are incompletely understood. By 2001, several studies suggested that a variety of signaling pathways converge on the extracellular signal-regulated kinase (ERD)/MAPK pathway to internalize NPT2a protein.[23] Yet, this signaling process insufficiently explains the integrated regulation of Pi homeostasis by the kidney. Rather, participation of protein/protein interactions in the regulatory process is highly likely, considering that the brush border membrane proteins Na^+Pi-Cap1 and NHERF-1 interact with NPT2a and result in protein release and internalization (see earlier).[14,24] The FGF23 and PTH signaling pathways appear to converge nonadditively on NHERF-1 to downregulate NPT2a.[25] Nevertheless, the interrelated mechanisms governing the abundance of NPT2a require further investigation.

3 PHOSPHATE REGULATION OF VITAMIN D METABOLISM

Pi is one of the three major factors that modulate 25(OH)D-1α-hydroxylase enzyme activity, the most important regulatory mechanism for activation of vitamin D.[26,27] In health, Pi depletion and resultant hypophosphatemia stimulate the activity of this enzyme and increase circulating levels of this hormone also called calcitriol. In contrast, Pi loading with consequent hyperphosphatemia inhibits formation of $1,25(OH)_2D$. However, the precise mechanism whereby Pi modulates this adaptive effect in health remains unknown. Hypophosphatemia due to Pi depletion, and hyperphosphatemia due to Pi loading, are associated with compensatory alterations in renal Pi transport that may mediate the changes in $1,25(OH)_2D$ production. These compensatory modifications are likely due to a direct effect of Pi depletion or loading on renal Pi transport. Indeed, a low-Pi diet stimulates transepithelial transport of Pi in murine renal proximal tubules, a change paralleled by an increased abundance of the apically localized Na/Pi cotransporter, NPT2a.[28] Pi regulation of vitamin D metabolism remains central to understanding and treating many of the disorders of Pi homeostasis.[29] The aberrant metabolism of vitamin D encountered in many heritable disorders of Pi homeostasis may be viewed as paradoxical. In virtually all disorders due to a primary abnormality in renal Pi transport, hypophosphatemia or hyperphosphatemia is associated with decreased or increased serum $1,25(OH)_2D$ levels, respectively. This suggests that the effect of Pi on 25-hydroxyvitamin D-1α-hydroxylase activity may occur indirectly and secondary to alterations in renal Pi transport systems. Therefore, it is not surprising that the prevailing serum $1,25(OH)_2D$ levels in disorders of renal Pi transport, such as XLH, tumor-induced osteomalacia (TIO), and tumoral calcinosis, display a highly significant positive correlation with the renal tubular maximum of Pi reabsorption per liter of glomerular filtrate rate (TmP/GFR).[30] This suggests that renal tubular reabsorption of Pi (TRP) is a major determinant of renal $1,25(OH)_2D$ production. Of course, this does not establish that 25-hydroxyvitamin D-1α-hydroxylase activity, in response to Pi depletion or loading, depends solely upon renal Pi transport. Mice with targeted disruption of the *Nherf1* gene manifest decreased apical membrane localized NPT2a receptors, renal Pi wasting, hypophosphatemia,[14] and inappropriately decreased serum $1,25(OH)_2D$ levels (Drezner, unpublished observations). In contrast, Tenenhouse et al.[31] concluded from studies of the $Npt2a^{-/-}$ mouse that NPT2a and renal Pi transport do not influence 25(OH)D-1α-hydroxylase activity. However, their data may have limited applicability for the role of renal Pi transport in vitamin D metabolism, because compensatory developmental changes often confound

studies in knock-out mice, even when there is absence of target protein function. For example, several studies[32,33] indicate that the Na^+/Pi cotransporter, NPT2c, normally expressed in the kidneys of young mice, has sustained activity in adult *Npt2a*$^{-/-}$ mice and is likely responsible for their residual renal Pi transport with only mild hypophosphatemia. Absence of NPT2c expression in the kidneys of adult normal and *Nherf*$^{-/-}$ mice shows that enhanced NPT2c expression in adult *Npt2a*$^{-/-}$ mice may represent a unique compensatory mechanism that limits this model for conclusions regarding regulation of vitamin D metabolism (Drezner, unpublished observations). In agreement, the studies of Segawa et al.[34] suggested that the unique enhancement of 25(OH)D-1α-hydroxylase activity in HHRH, compared to inhibitory effects in other renal Pi wasting disorders, may reflect a direct action of the NPT2c cotransporter on vitamin D metabolism.

The evident linkage between kidney TRP and $1,25(OH)_2D$ production indicates that improved understanding of the pathophysiology of the renal vitamin D regulatory system could offer novel treatment regimens for many disorders of Pi homeostasis.

4 THE HERITABLE RENAL PHOSPHATE WASTING DISORDERS

Starvation causing hypophosphatemia and renal failure causing hyperphosphatemia are likely the most common explanations for overt disruption of Pi homeostasis. However, it has been the heritable disorders of renal Pi transport that have been especially instructive concerning Pi regulation. Largely accounting for our improved understanding of renal Pi transport, a still increasing number of genes explain the heritable renal Pi wasting diseases (Table 40.1). However, their precise pathophysiology has been elusive because the hormonal/metabolic control of Pi homeostasis in the kidneys and bone remains incompletely understood. The direct link from the gene mutation to the downstream hormonal/metabolic abnormalities may be unknown. Fortunately, a complex series of studies performed in patients with TIO, an acquired hypophosphatemic disorder with features resembling the genetic Pi wasting diseases, uncovered a class of circulating factors, "phosphatonins," that regulate renal Pi transport and bone mineralization.[35–41] Thus, phosphatonin(s), when circulating in excess, could participate also in the many heritable disorders of renal Pi wasting, including autosomal dominant hypophosphatemic rickets (ADHR) (OMIM #193100), XLH (OMIM #307800), and autosomal recessive hypophosphatemic rickets (ARHR, types 1 and 2) (OMIM #241520 and #613312).[10] A common metabolic pathway could underlie many of these hypophosphatemic diseases. In fact, genetic analysis of these Mendelian disorders together with subsequent biochemical and animal studies revealed several novel molecules key for regulating renal Pi handling as well as bone mineralization.[29] These include FGF23, abundantly expressed in osteocytes in bone, and two bone-specific proteins, PHEX and dentin matrix protein 1 (DMP1), that regulate FGF23 expression. We now know that excessive FGF23 in the circulation is a prominent pathogenetic factor in ADHR, XLH, and ARHR, and thus the integrated mechanisms underlying this FGF23 excess have become increasingly understood.

The remainder of this chapter focuses on the clinical, biochemical, and genetic features of the most instructive heritable hypophosphatemic disorders, and presents current understanding of the molecular and hormonal mechanisms that govern their pathophysiology. In addition, we review accepted treatment strategies for these diseases, and new therapeutic agents under evaluation are discussed.

4.1 Autosomal Dominant Hypophosphatemic Rickets

4.1.1 Clinical Presentation and Diagnostic Aspects

ADHR, first described in 1971 by Bianchine et al.,[42] is particularly rare and characterized in full expression by hypophosphatemia due to renal Pi wasting, inappropriately low or normal circulating $1,25(OH)_2D$ levels relative to the serum Pi concentration, elevated serum alkaline phosphatase (ALP), and rickets/osteomalacia.[43] Renal function and serum calcium and PTH levels are normal, and there is no aminoaciduria.

ADHR is especially notable for its variable age of presentation and incomplete penetrance.[44] Approximately 50% of individuals harboring a mutation in the causal gene present clinically evident disease at ages 1–3 years. Generally, there is severe bowing of the lower extremities and radiographic evidence of rickets which, in some cases, is pronounced and prominent at costochondral joints. All affected subjects are hypophosphatemic for age, with serum Pi values usually 2.0–3.0 mg/dL from renal Pi wasting. In some, the hypophosphatemia and renal Pi wasting persist into adulthood, whereas in others these abnormalities remit. The other 50% of patients have delayed disease onset, ranging from 14–45 years of age. Presentation after puberty generally manifests as bone pain, fatigue, and/or weakness, but there is no history of rickets including lower limb deformities. Typically, biochemical abnormalities are evident at presentation, including hypophosphatemia (1.0–2.0 mg/dL) from renal Pi wasting, while serum calcium and PTH concentrations and renal function are normal. Serum ALP is variably elevated and pseudofractures and/or stress fractures are occasionally evident. Delayed presentation has been observed only in women, often soon after puberty or pregnancy and delivery (see later).

TABLE 40.1 Biochemistries and Genetic Characteristics in Renal Pi Wasting Disorders

	XLH	ADHR	ARHR	HHRH	McCune–Albright
Abnormal genes	***PHEX***	***FGF-23***	***DMP1***	***NPt2c***	***GNAS1***
Transmission	**X-linked dominant**	**Autosomal dominant**	**Autosomal recessive**	**Autosomal recessive**	**Postzygotic mutation**
FGF-23					
Serum FGF-23	N or ⇑	N* or ⇑	N* or ⇑	N or ⇓	⇑
Production	⇑	N or ⇑	⇑	N	⇑
Degradation	⇓	⇓	N	N	N
Bioactivity	N	N	N	N	N
P Homeostasis					
Serum P	⇓	⇓	⇓	⇓	⇓
Renal TmP/GFR	⇓	⇓	⇓	⇓	⇓
GI P absorption	⇓	⇓	⇓	⇑	⇓
Ca homeostasis					
Serum Ca	N	N	N	N or ⇑	N
Urine Ca	⇓	⇓	⇓	⇑	N
Nephrolithiasis	–	–	–	+	–
GI Ca absorption	⇓	⇓	⇓	⇑	?
Serum PTH	N	N	N	N	N
Vitamin D metabolism					
25(OH)D	N	N	N	N	N
1,25(OH)2D	N* or ⇓	N* or/⇓	N* or ⇓	⇑	N* or ⇓
Bone metabolism					
Serum alk. phos.	N or ⇑	N or ⇑	N or ⇑	N or ⇑	N or ⇑

N*: Inappropriately normal for the prevailing serum phosphorus concentration.
ADHR, Autosomal dominant hypophosphatemic rickets; alk. phos., alkaline phosphatase; ARHR, autosomal recessive hypophosphatemic rickets; *DMP1*, dentin matrix protein 1; *FGF-23*,fibroblast growth factor; HHRH, hypophosphatemic rickets with hypercalciuria; XLH, X-linked hypophosphatemia.

4.1.2 Genetic Abnormality

Using a large well-characterized ADHR kindred, Econs et al.[45] in 1997 reported a genome-wide linkage study to determine the chromosome location of the causal gene. Two-point LOD scores linked ADHR to the markers D12S314 and CD4. Multilocus analysis indicated that the gene locus was on chromosome 12p13 in the 18-cM interval between the flanking markers D12S100 and D12S397. In subsequent studies, a smaller ADHR family was added.[46] The two-point LOD score for marker D12S397 was 7.68 in the first family and 1.1 in the second. These investigators then screened for single recombination events that provisionally mapped the ADHR locus to 1.5 mB between D12S1685 and D12S1594. Using a positional cloning approach, which included annotation of 37 genes within 4 Mb, heterozygous causal missense mutations were eventually identified in the gene encoding FGF23.

FGF23 is expressed in various tissues, but most abundantly in bone, particularly in osteocytes and lining cells. The FGF23 protein is a 251 amino acid polypeptide including a 25 amino acid N-terminal signal sequence. After removal of the signal peptide, FGF23 appears as a 32 kDa protein on Western blots.

To date, several *FGF23* mutations causing ADHR (including R176Q, R176W, R179Q, and R179W) have been reported, each resulting in an amino acid change at $_{176}$RXXR$_{179}$/S$_{180}$, a subtilisin-like proprotein convertase (PC) consensus cleavage site.[46–50] These amino acid changes causing ADHR engender partial resistance of intact FGF23 to proteolytic cleavage[51] and thereby increase circulating FGF23 concentrations (see later).

4.1.3 Molecular Genetics and Animal Model

The seminal discovery in 2000 that mutated *FGF23* causes ADHR[46] provided the first link to understanding

the interwoven pathogeneses of the genetic hypophosphatemic diseases and the acquired disorder, TIO. In 2001, Shimada et al.,[52] seeking to identify the causative factor(s) underlying TIO, used recombinant cells to evaluate the biological activity of the secreted putative phosphatonins. Subcutaneous implantation of Chinese hamster ovary cells into nude mice, and consequent stable expression of the various factors, revealed that DMP1 and matrix extracellular phosphoglycoprotein (MEPE) hypersecretion resulted in neither increased renal Pi clearance nor hypophosphatemia. However, cells stably expressing FGF23 not only induced hypophosphatemia with increased renal Pi clearance, but also resulted in high serum ALP activity, low circulating $1,25(OH)_2D$, and histological evidence of impaired skeletal mineralization featuring widening of growth plates as well as osteomalacia. Thus, this continuous production of FGF23 in vivo reproduced key clinical, biochemical, and histological features of TIO. Notably, analysis of the recombinant FGF23 products produced by the Chinese hamster ovary cells indicated that the proteolytic cleavage of FGF23 that normally occurs at the RXXR motif was the site of the missense mutations in patients with ADHR. This suggested that the defects in FGF23 at ^{176}Arg and ^{179}Arg prevented such proteolytic cleavage.

Subsequent studies confirmed this supposition. In 2001, White et al.[51] introduced ADHR mutations into human *FGF23* cDNA clones by site-directed mutagenesis and transiently transfected them into HEK293 cells. Antibodies directed toward the C-terminal portion of FGF23 revealed that the native protein resolved as 32 kD and 12 kD species in the conditioned media, but the ADHR mutated FGF23 was detected only as the 32 kD band, consistent with lack of proteolysis. Similarly, an N-terminal FLAG-tagged native FGF23 resolved as two bands of 36 kD and 26 kD, whereas the mutant FGF23 resolved primarily as the 36 kD protein species. Confirmation of these observations utilized site-specific antibodies that showed cell-secreted, wild-type FGF23 was partially cleaved between ^{179}Arg and ^{180}Ser, whereas ADHR mutant FGF23 was partially resistant to cleavage.[50] This matched findings from intact animals representing a tumor-bearing nude mouse system.[52–54] FGF23 proteolysis is achieved by PC cleavage,[49,54] most likely PC2 and its cofactor 7B2.[54] Hence, these data, in concert with those documenting that FGF23 bioactivity requires an intact protein, indicated ADHR mutations protect FGF23 from proteolysis, thereby elevating the circulating concentrations of intact FGF23 and leading to Pi wasting (Fig. 40.2A). ADHR is caused by *FGF23* gain-of-function mutation that increases the biological potency of FGF23.

Although these in vitro and animal studies predicted that the ADHR phenotype is caused by elevated circulating concentrations of cleavage-resistant FGF23, they provided no explanation for the variable expressivity of this disease. Then, additional studies[55] showed that, remarkably, serum FGF23 concentrations are not consistently elevated in individuals who could be called "carriers" of ADHR. Rather, FGF23 concentrations were elevated only when active disease was present. Furthermore, patients in ADHR remission, despite their prior history of clinical manifestations, also had normal serum FGF23 levels. Thus, they showed correction of Pi metabolism by modulating their FGF23 level, rather than somehow compensating for its effects.[55]

While the explanation for the variably modulated serum FGF23 concentration in ADHR was not immediately apparent, it was striking that delayed onset of the disease occurs primarily in girls during puberty and women following pregnancy,[44,51,55,56] situations that predispose to iron deficiency.[56–59] In fact, serum FGF23 was found to correlate negatively with circulating ferritin,[60] and reduced serum iron concentrations to correlate strongly with increased serum FGF23 in ADHR patients.[56] Mice harboring the orthologous ADHR *R176Q-Fgf23* mutation supported a role for iron in the modulation of circulating FGF23 levels in ADHR.[61] *Fgf23* mRNA was stimulated robustly in bone during iron deficiency, but the alteration could be counteracted in wild-type mice by enhanced proteolytic cleavage of the excess FGF23. However, in ADHR mice carrying the *R176Q-Fgf23* mutation, the compromised proteolytic step in FGF23 processing led to increased circulating levels of the hormone. These findings indicated a mechanistic link between iron and Pi homeostasis that explained, in part, the delayed onset of ADHR and the variably modulated serum FGF23 concentration. Nevertheless, how iron deficiency increased FGF23 production remained unknown. Moreover, chronic *Fgf23* mRNA upregulation might stimulate *R176Q-Fgf23* processing at domains other than the $_{176}$RXXR$_{179}$/S$_{180}$, PC site, or by other mechanisms. Nevertheless, ADHR clearly involves gene–environment interactions, that perhaps offer novel therapeutic targets.

4.1.4 Genetic Testing

The clinical presentation of ADHR, XLH, and ARHR in children is similar. Thus, until recent availability of genetic testing, diagnosing each depended largely on discerning the pattern of inheritance. Male-to-male transmission does exclude XLH, and the disease in a parent excludes ARHR. However, there is often insufficient information to establish among the disease types by analysis of the inheritance pattern. Establishing the correct diagnosis is needed for accurate genetic counseling and to direct unique therapeutic intervention, such as iron supplementation. The choice of medical therapy for most renal Pi wasting disorders is combination treatment with Pi and $1,25(OH)_2D$, and this could apply to patients with ADHR. However, there have been isolated reports of successful treatment of ADHR with vitamin D or $1,25(OH)_2D$ alone, yet the studies discussed earlier suggest iron therapy

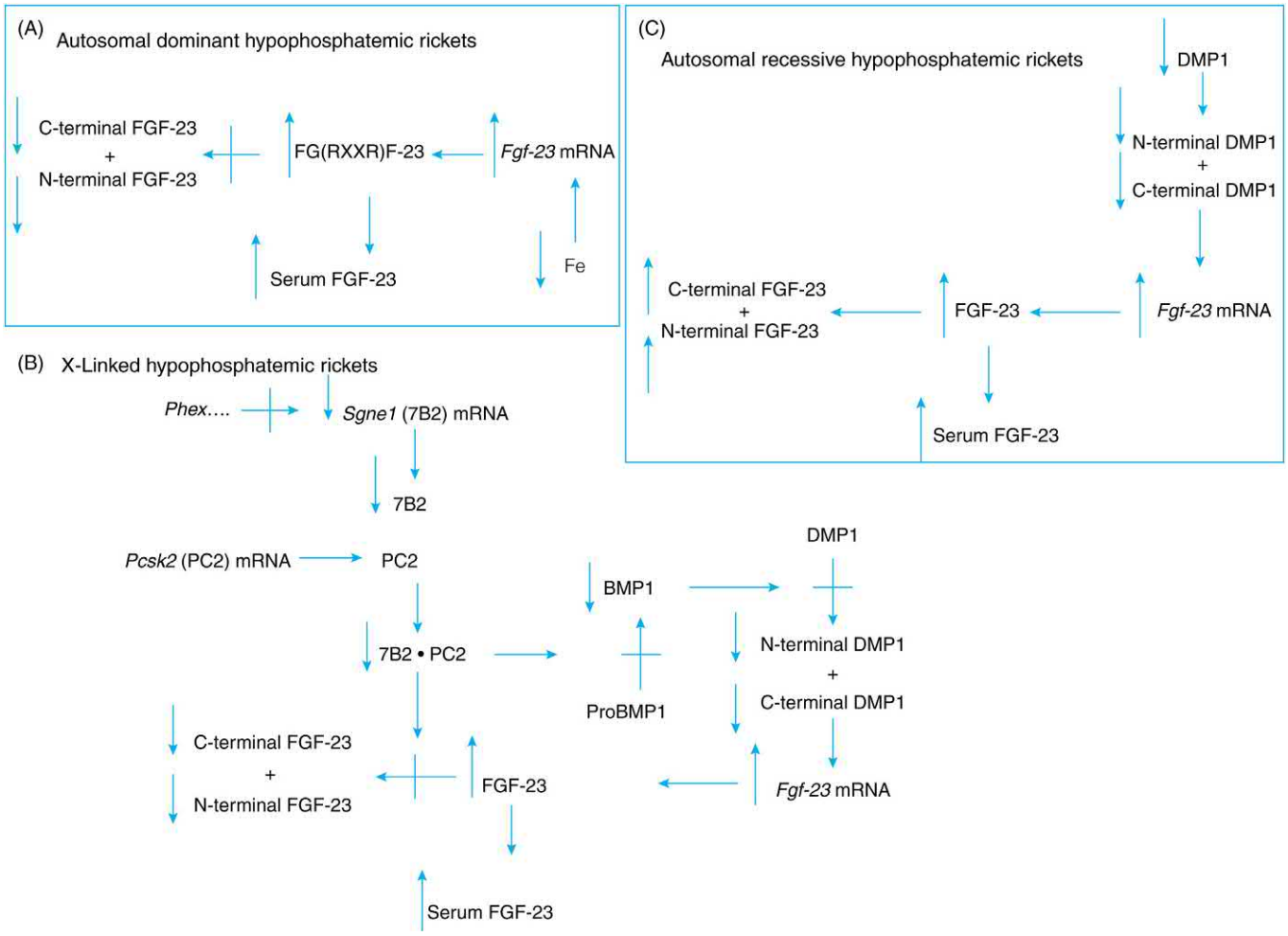

FIGURE 40.2 An overview of the abnormalities resulting in an increased serum FGF23 concentration in autosomal dominant hypophosphatemic rickets (ADHR), autosomal recessive hypophosphatemic rickets (ARHR), and X-linked hypophosphatemia (XLH). (A) In ADHR, a genetic defect in the RXXR motif of FGF23 limits 7B2·PC2 proteolysis of FGF23, leading to increased circulating FGF23. Contributing to this abnormality can be the stimulatory effect of decreased iron concentration on Fgf23 mRNA production. (B) In XLH, decreased 7B2•PC2 activity directly limits FGF23 proteolysis and, through a complex process involving regulation of active BMP1 production and DMP1 cleavage, indirectly enhances Fgf23 mRNA. These changes increase the circulating concentration of FGF23. (C) In ARHR, reduced production of the C-terminal DMP1 cleavage product increases Fgf23 mRNA production and enhances the production and serum concentration of FGF23.

might lead to remission in selected patients. Thus, unambiguous diagnosis of ADHR is beneficial. Genetic testing for ADHR is now commercially available. Also, numerous research laboratories worldwide can perform mutation analysis of *FGF23*. ADHR is, however, extremely rare. In our ongoing study, including mutation analysis of 284 children with hypophosphatemic rickets that we care for at Shriners Hospital for Children, St. Louis, MO, USA, we have yet to encounter ADHR or ARHR.[62]

4.1.5 Treatment

Prior to treating ADHR, baseline studies needed for follow-up include: (1) assay of fasting or preprandial serum phosphorus, calcium, ALP, creatinine, PTH, 25(OH)D, $1,25(OH)_2D$, FGF23, and iron; (2) 24-h urine collection to determine calcium and Pi excretion corrected for creatinine content, and TRP and TmP/GFR; and (3) radiographs to assess severity of rickets and long bone deformities, and to detect any fractures. A detailed assessment of the family supported by laboratory investigations, including mutation analysis, could be beneficial. Iliac crest biopsy may be necessary under certain circumstances, including poor response to treatment or markedly abnormal bone radiographs. Collectively, these studies will characterize the disorder and provide the basis for then assessing therapeutic interventions.

Only case reports discuss the therapy of ADHR. Approaches have included large doses of vitamin D, or combination therapy with Pi and either vitamin D or $1,25(OH)_2D$. Generalized intermittent bone pain, diffuse weakness, and fatigue have responded to high doses of vitamin D and, in some cases, serum phosphorus levels have increased to normal with healing of rickets documented radiographically.[44,55] In general, however,

ADHR and other hypophosphatemic disorders caused by genetic mutations associated with low or inappropriately normal $1,25(OH)_2D$ levels are treated with oral Pi and $1,25(OH)_2D$.[63] Details about such combination drug management are best established for XLH, the most common heritable hypophosphatemic disorder (see later). For iron deficient ADHR patients, it may be possible to stop Pi and $1,25(OH)_2D$ treatment if iron status is optimized.[64] Notably, important therapeutic successes for XLH are being reported using the investigational human anti-FGF23 monoclonal antibody KRN23 (see later), but this biologic (burosumab) has not been tested in ADHR.

4.2 X-Linked Hypophosphatemia

4.2.1 Clinical Presentation and Diagnostic Aspects

XLH, the prototypic and most prevalent heritable renal Pi wasting disorder, features rickets causing skeletal deformities, and growth retardation in children and osteomalacia with enthesopathy and joint disease during adult life.[65–67] XLH is an X-linked dominant disorder with complete penetrance of renal Pi wasting and consequent hypophosphatemia. However, its expressivity varies from mild with apparently isolated hypophosphatemia due to renal Pi wasting,[62] to severe with clinically obvious skeletal disease.[68] In children, the common evident manifestations include short stature and a variety of lower extremity deformities (Fig. 40.3). The short stature is conditioned primarily at the lower limbs that have the fastest growing long bones before puberty. Most children with XLH manifest widened wrists and/or knees secondary to the metaphyseal flaring of rickets (Fig. 40.4). Sometimes there is seemingly paradoxical premature closure of the cranial sagittal suture causing dolichocephaly (Fig. 40.5). Additional signs of XLH can include late dentition, tooth abscesses stemming from poor mineralization of the interglobular dentin, and premature closure of all cranial sutures. However, these features do not emerge until 6–12 months of age or older.[69] Histological study of XLH bone reveals osteomalacia without osteopenia, and rickets if a growth plate is sampled.[66] Typically, serum 25(OH)D levels are unremarkable, but unsupplemented $1,25(OH)_2D$ levels are inappropriately normal relative to the prevailing hypophosphatemia with decreased renal 25(OH)D-1α-hydroxylase activity.[70–72] Studies in *hyp*- and *gy*-mice, murine homologs of XLH, have shown that the aberrant regulation of this hydroxylase is confined to the proximal convoluted tubule, the site of the abnormal Pi transport.[73–75]

4.2.2 Genetic Abnormality

XLH is caused by one of many loss-of-function defects now documented for the gene designated *PHEX* (*Ph*osphate regulating gene with homologies to *E*ndopeptidases located on the *X*-chromosome). In 1986, Read et al.[76] and Machler et al.[77] reported linkage of XLH to DNA probes DXS41 and DXS43, which mapped to Xp22.31-p21.3. Subsequently, Thakker et al.,[78,79] reported linkage to DXS197 and DXS207 and, using multipoint mapping techniques, determined the most likely order of the markers to be Xpter-DXS85-(DXS43/DXS197)-*HYP*-DXS41-XcenandXpter-DXS43-*HYP*-(DXS207/DXS41)-Xcen. The relatively few informative pedigrees available then permitted identification

FIGURE 40.3 (A) This 10-year-old boy with XLH shows, despite medical treatment, characteristic bowing (genu varum) of his lower limbs. (B) This 18-year-old woman, untreated medically or surgically life-long for XLH, shows severe "wind swept" deformity of her lower extremities. (C) This girl with XLH has characteristic "knock-knee" (genu valgum) deformity of her lower extremities.

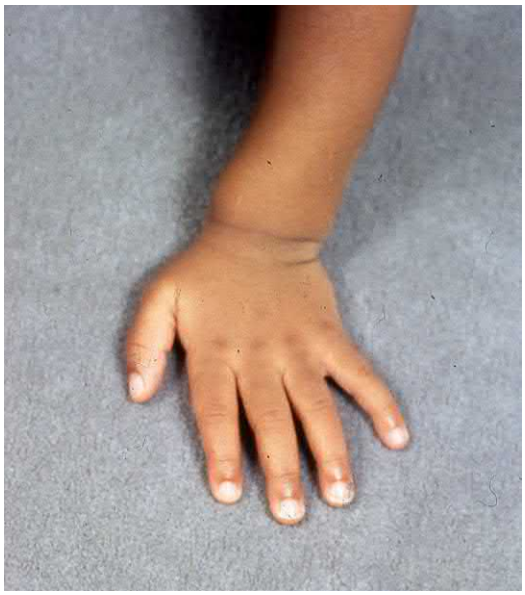

FIGURE 40.4 The left wrist of this young girl about to begin medical treatment for XLH shows characteristic "flaring" from widened metaphyses.

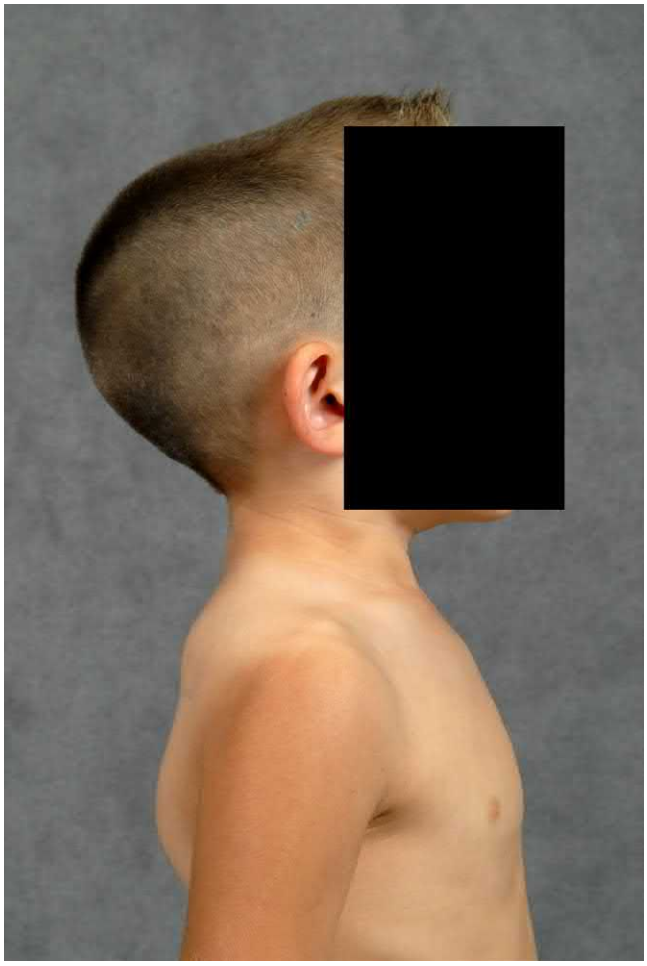

FIGURE 40.5 This 3-year-old boy with XLH has characteristic dolichocephaly from premature closure of his cranial sagittal suture.

of flanking markers 20 cM apart. Independent and collaborative efforts of the HYP consortium investigated 13 multigenerational pedigrees and refined the mapping of the Xp22.1-p21 region. Construction of a YAC contig spanning the *HYP* gene region identified markers 350 kb apart on a single YAC. Subsequently, a cosmid contig spanning the *HYP* gene region led to discovery of deletions permitting characterization of cDNA clones that mapped to cosmid fragments in the vicinity of the deletions. Database searches with these cDNAs detected homologies at the peptide level to a family of endopeptidases. In 1995, these efforts established *PHEX* as the gene responsible for XLH.[80]

PHEX encodes a 749 amino acid protein consisting of three domains: (1) a small N-terminal intracellular tail; (2) a single short transmembrane domain; and (3) a large C-terminal extracellular domain containing the 10 conserved cysteine residues and a HEXXH pentapeptide motif typical of zinc metalloproteases.[81] PHEX homology with metalloproteases led to its inclusion in the M13 family of membrane-bound metalloproteases, also called neutral endopeptidases (NEPs).[82–84] M13 family members, including NEP 24.11, endothelin-converting enzymes 1 and 2, the Kell blood group antigen, neprilysin-like peptide (NL1), and endothelin converting enzyme-like, degrade or activate a variety of peptide hormones. Conservation within the PHEX structure of catalytic glutamate and histidine residues (equivalent to Glu^{648} and His^{711} of NL1) argued for similar protease activity, as did alignment of PHEX mutations with regions required for peptidase activity in NL1.[85] Furthermore, like other NEPs, immunofluorescence studies revealed a cell-surface location for PHEX in an orientation consistent with a type II integral membrane glycoprotein.

Cloning of the PHEX gene led relatively rapidly to cloning of the homologous murine *Phex* gene and then identification in the late 1990s of mutations in the murine homologs of XLH, *hyp*- and *gy*-mice.[86–88] Unlike 97% of known genes, neither the human nor the murine gene causing XLH has a Kozak sequence, a purine at the −3 position before the ATG initiation sequence. As many such genes are regulated posttranscriptionally, this anomaly, which limits transcription, may impact the hormonal and metabolic regulation of *PHEX/Phex*.

Investigation of murine tissues and cell cultures indicated *PHEX* is predominantly expressed in bones and teeth,[86,88–92] while its mRNA, protein, or both, have also been found in lung, brain, muscle, gonads, skin, and parathyroid glands.[85,93] Neonatal and adult mouse studies revealed that the cells in bone and teeth containing PHEX are the osteoblast (OB)/osteocyte and the odontoblast/ameloblast, respectively. PHEX in such cells is at the surface membrane, endoplasmic reticulum, and Golgi compartment. Notably, *PHEX* expression is absent in the viscera including the kidney, hepatocytes, intestine, and cardiac and skeletal muscle. The ontogeny of PHEX expression reveals that the protein appears in OBs at both primary and secondary ossification centers, suggesting a role in mineralization.

To date, hundreds of *PHEX* mutations consisting of deletions, frameshifts, insertions, and duplications, as well as splice site, frameshift, nonsense, and missense defects, have been documented in XLH.[94–96] They are scattered throughout exons 2–22, which encode the 749 amino acid residue extracellular domain. Early on, *PHEX* mutations were identified in >90% of probands from selected kindreds in which an evident X-linked dominant pattern of inheritance was present. In sporadic cases of hypophosphatemic rickets, for which no a priori family history was present, as many as 70% of subjects, who passed the disease to their children in an X-linked dominant inheritance pattern, had demonstrable *PHEX* mutations. The great majority of *PHEX* mutations lie within the 3′ end of the gene, at exons 14–22, indicating this site encodes a critical region. When, early on, *PHEX* coding region mutations were not detected in ~35% of patients, Christie et al.[97] showed intronic mutations were possible that result in mRNA splicing abnormalities. Now, *PHEX* mutation/deletion analysis is readily available from several fee-for-service laboratories, and XLH patients are expected to show a positive result. However, single mutations have also been identified within the 3′ and 5′ untranslated regions of *PHEX*.[98] In 2014, Mumm et al.[62] reported a 3′-UTR mutation (c.*231A > G) near the polyadenylation signal[99] that is a relatively common and mild American mutation that masquerades clinically as sporadic or X-linked recessive hypophosphatemic rickets/osteomalacia. Although all *PHEX* mutations in XLH cause loss of function, the mechanism(s) is unclear. Missense defects may interfere with protein trafficking, resulting in protein sequestration in the endoplasmic reticulum.

To confirm that diminished *PHEX/Phex* expression in OBs initiates the pathogenesis of XLH, several investigators used targeted overexpression of *Phex* trying to normalize OB mineralization in vitro and to reverse the *HYP* phenotype in vivo. Surprisingly, these studies[100,101] revealed that restoration of *Phex* expression and enzymatic activity using immortalized *hyp*-mouse OBs did not restore mineralization in vitro. In complementary studies, Liu et al.[100] and Bai et al.[102] found transgenic *hyp*-mice (*Osc-Phex-Hyp; pOb2.3[ColIaI]-Phex-Hyp*), despite expressing abundant *Phex* mRNA and enzyme activity in mature OBs and osteocytes, exhibited mild hypophosphatemia and persistently abnormal vitamin D metabolism. Moreover, although exhibiting a modest improvement in bone mineralization, osteomalacia persisted similar to nontransgenic *hyp*-mice. This was probably due to a disparity between the temporal and developmental *Osc* and *pOb2.3* promoter-driven *Phex* expression versus endogenous regulation of *Phex*. Perhaps, the transgenic animals experienced delayed *Phex* expression or expression in different OB-related cell subpopulations compared to normal animals. In fact, neither of the promoters was expressed in the preosteoblast, and the osteocalcin promoter appeared at least 4 days later than Phex in control OBs. Possibly, the delayed expression of Phex activity was unable to overcome OB dysfunction established early in development, resulting in impairment of mineralization. Regardless, these observations did not exclude a role for inadequate *Phex* expression in the genesis of XLH. Indeed, subsequent studies confirmed this and established the OB (and osteocyte) as the likely site(s) for the pathophysiological relevant disturbance in XLH. Using mice with conditional osteocalcin-promoted (*OC*) *Phex* inactivation in OBs, Yuan et al.[103] in 2008 showed that *Oc-Cre-Phex*Δflox/y mice exhibited biochemical and bone histological abnormalities indistinguishable from those in *hyp*-mice. This provided compelling evidence that aberrant PHEX function in OBs alone is sufficient to cause the *HYP* phenotype. In 2017, Boukpessi et al.[104] demonstrated that osteopontin, an inhibitor of mineralization normally degraded by PHEX, accumulated at sites of defective mineralization near osteocytes and tooth dentin in XLH, and likely contributed to the dentoosseous pathobiology. Investigators generally agree, however, that the primary pathogenic error of XLH is the impaired renal proximal tubule reabsorption of Pi.

4.2.3 Molecular Genetics and Animal Models

The consequence of *Phex* mutation can be demonstrated directly in the brush border membrane of the proximal nephron in *hyp*-mice. Early on, it was controversial whether this renal abnormality was primary or

secondary to the elaboration of a hormonal factor. In support of a primary kidney abnormality, renal tubule cells in primary culture from *hyp*-mice were persistently defective in Pi transport,[105,106] likely due to decreased expression of *Npt2a*.[107,108] In contrast, however, impaired renal Pi transport by intact or parathyroidectomized normal mice, after parabiosis to *hyp*-mice, implicated a humoral factor.[109] Additional studies provided compelling evidence that the renal Pi transport defect in XLH is due to a circulating hormone or metabolic factor. In fact, immortalized cells in culture from the renal tubules of *hyp*- and *gy*-mice exhibited normal Na^+-Pi transport, suggesting that the effects observed in primary cultures actually represented "impressed memory" but not an intrinsic abnormality.[110,111] Additional strong evidence for a humoral pathogenesis in XLH came from cross-transplantation of kidneys of normal and *Hyp*-mice. This resulted in neither transfer of the mutant phenotype nor its correction, and established the humoral basis for XLH.[112] Localization in 1994 of the gene encoding the Na^+-Pi cotransporter to autosome 5 substantiated the conclusion that the defect in Pi transport in XLH was not intrinsic to the kidney.[113] These studies were supported by data indicating *hyp*-mouse OBs produce both phosphaturic and mineralization inhibitory factors.[114] Subsequent investigations argued that a circulating factor(s), that is, phosphatonin(s), acted importantly in the pathophysiological cascade causing XLH, and focused on the identification and characterization of their biological activities.

Elucidating the role of FGF23 in ADHR provided a link for integrating the pathogeneses of the hypophosphatemic diseases. Identification of high circulating levels of FGF23 underlying XLH established this association for the heritable disorders. Initial studies of XLH patients and the *hyp*-mouse had identified abnormal production or elevated circulating levels of several phosphatonin(s), including FGF23, sFRP4, MEPE, and FGF7.[103] However, further investigations established it was FGF23 that played a singular role in the regulation of Pi homeostasis in XLH. The data that supported this conclusion included: (1) transgenic mice overexpressing FGF23 under the control of the α1(I) collagen promoter exhibited growth retardation, osteomalacia, and disturbed Pi homeostasis consistent with XLH and with *hyp*-mice;[48,115] (2) XLH patients[116,117] and *hyp*-mice[118] had increased circulating levels of FGF23; (3) elevated blood levels of FGF23 in *hyp*-mice reflected enhanced production of FGF23 and inhibited degradation of full-length FGF23;[103] (4) deletion of *Fgf23* from *hyp*-mice reverses the *HYP* phenotype;[118] and (5) selective deletion of *Phex* in OBs increases circulating FGF23 (but not other phosphatonins), and in mice engenders renal and bone abnormalities characteristic of XLH.[103] These observations established that increased bone production and serum levels of MEPE, sFRP4, and FGF7 were not crucial for developing the classical *HYP* phenotype, whereas increased bone production and decreased proteolysis of FGF23 with consequent elevation of circulating FGF23 concentrations seemed requisite. Collectively, these data suggested FGF23 is the phosphatonin pivotal to the pathogenesis of XLH as well as ADHR.

Despite these advances, how inactivating mutations of *PHEX* enhance *FGF23/Fgf23* mRNA production and decrease FGF23 proteolysis remained elusive. Extensive characterization of the *PHEX/Phex* gene and PHEX protein in humans and mice repeatedly failed to identify either a substrate for PHEX[115,119–126] or the downstream effects of PHEX causing the classical renal and bone findings of XLH.[53,122,127,128] In fact, by the early 2000s, several studies excluded FGF23 as a substrate for PHEX.[119–121] Investigations to establish that changes in circulating Pi mediate alterations in serum FGF23, and thereby putative hormone degradation or production, were variable and inconclusive.[129–133] Thus, therapeutically altering FGF23 to treat several human diseases, through modification of PHEX interaction with FGF23 or an alternative substrate, was not possible at that time.

Then, downstream PHEX-dependent effects that modulate FGF23 production and degradation were elucidated. Not only did FGF23 production and degradation occur primarily in OBs/osteocytes, but also this was seemingly regulated by 7B2 and PC2, and the activity of the heterodimer 7B2·PC2. Yuan et al.[54,134] documented that a decrease in the 7B2 chaperone protein mRNA in *hyp*-mouse OBs, and consequent diminished 7B2·PC2 enzyme activity, enhanced *Fgf23* mRNA production and limited FGF23 degradation. Enhanced FGF23 production was mediated by a downstream effect of decreased 7B2·PC2 enzyme activity and impaired DMP1 degradation leading to deficiency of the 57 kDa C-terminal proteolytic DMP1 fragment, which increases *Fgf23* mRNA (Fig. 40.2B).[135] While these observations in *hyp*-mouse OBs supported that 7B2-dependent PC2-mediated alterations in FGF23 production and degradation are pathogenic and likely downstream PHEX-dependent effects, their role in generating the *HYP* phenotype remained unclear.

However, subsequent treatment of *hyp*-mice with hexa-D-arginine, a pharmacological agent that increases 7B2 mRNA and hence 7B2·PC2 enzyme activity, confirmed this pathway as central to the genesis of XLH. Treatment not only restored 7B2·PC2 activity, by increasing 7B2 production, but also normalized the *HYP* phenotype, correcting Pi and vitamin D homeostasis, as well as bone modeling and mineralization.[136] While much work remained to establish the cellular and molecular mechanism regulating PHEX–7B2 interactions, the findings provided novel insight into the biosynthetic mechanisms modulating FGF23 synthesis and created a previously unrecognized therapeutic target for treatment of XLH. In 2014, evidence emerged in XLH that

iron deficiency might alter FGF23 cleavage perhaps contributing to the abnormal set-point for Pi.[137] In 2016, Murali et al.[138] showed that excessive osteocytic Fgf23 secretion contributes to accumulation of inorganic pyrophosphate and the mineralization defect in *Hyp* mice.

4.2.4 Genetic Testing

Clinical presentation of the familial hypophosphatemic disorders can be remarkably similar. Hence, diagnosing XLH has routinely depended upon ascertaining, where possible, a pattern of inheritance consistent with X-linked dominant transmission. XLH is not passed from affected fathers to their sons, but is transmitted to all of their daughters. Affected mothers pass on XLH with 50/50 probability to each son and daughter. Hence, XLH is more prevalent in females than in males. Male-to-male transmission excludes XLH, and presence of the disease in a parent essentially excludes ARHR (see later). In some instances, females are emphasized to have relatively mild XLH. However, in 1996, we found no evidence for gender, race, anticipation, or parent-of-origin effects on XLH expression in children.[139] Spontaneous mutation causing XLH is relatively frequent. Definitive diagnosis by *PHEX* mutation analysis is needed: (1) if genetic counseling is requested, while keeping in mind the relatively benign and increasingly treatable nature of XLH, and ii) optimal understanding of pharmacologic interventions. Since the 1970s, therapy for XLH has involved treatment with Pi and $1,25(OH)_2D_3$ in the USA[140] or 1α-hydroxyvitamin D_3 in some other countries. Other renal Pi wasting disorders, particularly ADHR, may require less aggressive use of this regimen or therapy with alternative drugs, such as iron (see previously). Hence, unambiguous diagnosis of XLH may be beneficial. Now, genetic testing (including exome, splice site, and deletion analysis) to diagnose the heritable disorders of renal Pi wasting is commercially available from a number of laboratories. Additionally, many research laboratories perform mutation analysis of the *PHEX* gene. A positive result can be expected in XLH.

4.2.5 Treatment

Decades ago, physicians used pharmacologic doses of vitamin D alone attempting to treat XLH. However, long-term observations indicated little control over the disease (vitamin D-resistant rickets) and this approach posed the serious problem of vitamin D intoxication with renal damage. Subsequently, therapy for XLH was influenced by better understanding of its pathogenesis, and this led to considerable successes especially in pediatric patients. Nevertheless, the desirability of introducing or extending current medical therapy for adults with XLH is less well understood. In adult life, growth is complete and the likelihood of skeletal deformity is diminished compared to pediatric years, yet osteomalacia persists.[65–67] Treatment for children currently aims to improve skeletal deformity, short stature, and bone pain. Medical therapy addresses the characteristic combined circulating deficiencies of $1,25(OH)_2D$ and Pi.[140] Generally, the regimen involves titration with $1,25(OH)_2D$ to overcome the "vitamin D resistance" and assure adequate absorption of dietary calcium in face of Pi supplementation.[140] The dose of calcitriol can be 1–2 μg/day given in two divided doses, and phosphorus 1–2 g/day, given in four to five divided doses.[141,142] Such combination therapy often improves the rickets and lower extremity deformities. Perhaps it also helps dental health. However, its effect on growth has been controversial, with some investigators reporting improvements in growth velocity, while others observed inconsistent or nonsignificant catch-up growth. Moreover, variable effects have been reported on measured or predicted adult height.[143] This treatment risks toxicity including abnormalities of calcium homeostasis, most notably secondary hyperparathyroidism that may become autonomous (tertiary hyperparathyroidism) and require parathyroidectomy. Renal toxicity is also possible, but normal kidney function in XLH despite long-standing medullary nephrocalcinosis has been reported.[144,145] Thus, appropriate monitoring of Pi and calcitriol dosage, especially often during growth, is mandatory to assess both for efficacy and safety. Now, pediatric orthopedists are increasing helpful with their use of epiphysiodesis (growth plate clamping) employing "staples" or more recently "eight plates" (Fig. 40.6) to mechanically straighten lower extremities possible only during growth. Need for osteotomy is diminishing from such improved medical and orthopedic care.

Perhaps the vitamin D receptor (VDR) promoter genotype can predict growth in children with XLH.[143] In one report, more than one-third of treated patients had a VDR Hap1− promoter genotype and severe growth defects, with 86% attaining an adult height at or below −2 SD. In contrast, only 11% of treated patients with a VDR Hap1+ promoter genotype had an adult height below −2 SD. The height burden from Hap1− status was evident before treatment, as early as the third to fourth year-of-life, and likely relates to the refractoriness to treatment of children presenting below the fifth percentile for height. For that reason, recombinant growth hormone has sometimes been given for short stature in pediatric XLH.[146]

More recently, calcimimetics as adjuvant therapy has been advocated for XLH.[147,148] Some add cinacalcet to the traditional regimen to abrogate the not infrequent Pi-induced increase in circulating PTH concentration seen as exacerbating the renal Pi transport defect (TmP/GFR). Perhaps this permits lower doses of Pi and calcitriol. Consequently, the incidence of secondary and tertiary hyperparathyroidism and nephrocalcinosis, known complications of standard therapy, may decrease.[148]

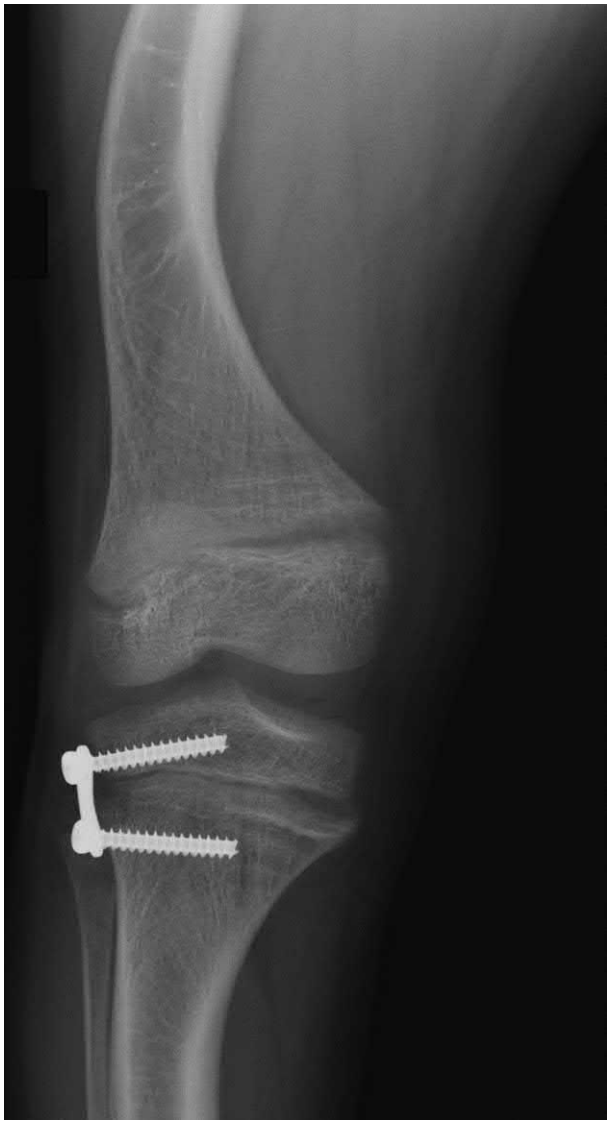

FIGURE 40.6 An "eight-plate" has been placed in the lateral proximal tibia to "clamp" endochondral bone formation at the lateral physis to mechanically straighten the lower extremity.

Now, investigation of the human anti-FGF23 monoclonal antibody KRN23 (burosumab) administered subcutaneously to children and adults with XLH is showing favorable clinical, biochemical, and radiographic results with a good safety profile.[149,150]

4.3 Autosomal Recessive Hypophosphatemic Rickets

4.3.1 Clinical Presentation and Diagnostic Aspects

Several people have manifested clinical, biochemical, and histomorphometric features of XLH or ADHR yet their family pedigrees, including known or suspected consanguinity, indicated autosomal recessive inheritance effectively excluding XLH and ADHR.[151–154] In subsequent studies, homozygous mutations in the gene encoding DMP1, a noncollagenous phosphoprotein, were associated with this entity. Symptoms of ARHR present in late infancy and include short stature, bowing of the lower extremities, and a rachitic rosary. There is hypophosphatemia from impaired renal TRP (TmP/GFR), and high-normal-to-moderately-elevated serum ALP activity, yet normal levels of circulating calcium and PTH and urinary calcium. Serum $1,25(OH)_2D$ levels are inappropriately normal relative to their hypophosphatemia, and serum FGF23 concentration is in the upper range of normal or elevated. Radiographs of the lower extremities generally exhibit metaphyseal cupping and fraying, and bowing of the femurs. Bone biopsies reveal severe osteomalacia and increased bone volume with excess osteoid not only on mineralized bone surfaces, but also in the lacunar regions ('halos) around embedded osteocytes like those observed in XLH. Some patients develop osteosclerosis and bone overgrowth.[155] Clinical and biochemical abnormalities are variable, but seemingly related to specific DMP1 mutations or patient age when the disease is expressed (see later).

4.3.2 Genetic Abnormality

Discovery of the etiology of ARHR was based on studies of the *Dmp1*-null mouse (see later), and initially utilized a DMP1 candidate gene approach with direct sequence analyses. In two ARHR families studied in 2006, loss-of-function *DMP1* mutations were revealed and featured: (1) homozygous deletion of nucleotides 1484–90 in *DMP1* exon 6, which resulted in a frameshift that caused loss of the wild-type stop codon, translation of nucleotides within the 3'-UTR, formation of a new stop codon, and replacement of the conserved C-terminal 18 residues with 33 unrelated residues (together causing loss of the highly conserved DMP1 signal peptide); and (2) a homozygous nucleotide substitution in the *DMP1* start codon (ATG to GTG), which substituted the initial methionine with valine (c.1 A > G, p.M1V), predicting loss of the highly conserved 16-residue DMP1 signal sequence.[152] Soon after, Lorenz-Depiereux and coworkers [151] identified two additional homozygous loss-of-function mutations, characterized by: (1) a 1-bp deletion in exon 6 (362delC) leading to a premature stop codon after generating 120 unrelated amino acids; and (2) a mutation in the canonical splice acceptor sequence (55-IG→C) of intron 2. The biochemistries and bone histomorphology from *DMP1* mutations 1484–1490del, 362delC, and 55-IG→C were typical of the ARHR described earlier. However, in 2009, Farrow et al.[153] reported that subjects harboring the large biallelic deletion of *DMP1* (c.1 A > G, p.M1V), which removed large portions of the gene, suffered from not only characteristic marked hypophosphatemia, persistent osteomalacia, and stunted growth, but also nerve deafness, facial and dental abnormalities, and learning disabilities suggesting that the

features of ARHR could be variable and perhaps mutation specific. Indeed, Turan et al.,[156] in 2010 described a family with ARHR, caused by a novel homozygous frameshift mutation (c.485del; p.Glu 163ArgfsX53) in exon 6 that resulted in a premature stop codon, in which affected children had a unique phenotype including biochemical and radiographic findings typical of rickets, plus other bone and tooth abnormalities featuring: (1) shortening and broadening of the metacarpals, and the proximal and distal phalangeal bones; (2) wide pulp chambers and thin dentin in unerupted normal-size and shaped teeth, and roots; and (3) diminished enamel thickness in erupted teeth, indicating rapid posteruption attrition. Also in 2010, Mäkitie et al.[155] described a family with a novel mutation of the splice acceptor junction of *DMP1* exon 6 (IVS5-1 G > A). This defect, in association with several downstream cryptic splice acceptor sites, likely altered pre-mRNA splicing and shifted the open-reading frame so that, if the resulting message was stable, there would be severely compromised DMP1 production. Individuals older than age 60 years carrying this mutation had skeletal abnormalities similar to, but more severe than, those reported by Turan et al.[156] These included not only osteomalacia, but also a progressive skeletal phenotype characterized clinically by short stature, joint pain, contractures, and immobilization of the spine, and radiographically by short and deformed long bones, significant cranial hyperostosis, enthesopathies, and calcifications of the paraspinal ligaments. Similar to the patients with the large biallelic deletion of *DMP1* (c.1 A > G, p.M1V), they also had hearing impairment and dental anomalies resulting in abscess formation. Interestingly, individuals heterozygous for this *DMP1* mutation manifested mild hypophosphatemia and focal osteomalacia without clinical evidence of skeletal dysplasia, representing the first expression of a mild ARHR phenotype in subjects carrying only a monoallelic *DMP1* mutation. Whether the earlier differences in expressivity are mutation-specific and/or age-dependent remains unknown.

Reportedly, DMP1 (expressed mainly in hypertrophic chondrocytes, OBs, and osteocytes) has multiple functions in postnatal skeletal development including chondrogenesis and growth plate and epiphyseal development. Not surprisingly, therefore, the *Dmp1*-null mouse has a chondrodysplasia-like phenotype that appears to be completely penetrant but manifests several days to weeks postnatally.[152,157] Their growth plates are expanded and disorganized, and endochondral ossification is decreased. Metaphyses fail to lengthen normally, and metaphyseal ends are wide. Furthermore, epiphyseal formation and calcification are delayed.[158] Collectively, these observations suggest that increased proliferation of chondrocytes, impairment in chondrocyte programmed cell death, poor calcification of cartilage matrix, and delayed blood vessel invasion contribute to the chondrodysplastic defect of *Dmp1*-null mice. These murine skeletal abnormalities appear to worsen with age, perhaps reflecting the variable presentation of ARHR with age-dependent expression of wide ranging skeletal abnormalities and associated conditions.

Nevertheless, this inconsistent disease penetrance may involve undetermined genetic modifiers linked to DMP1. In this regard, a patient with homozygous deletion of nucleotides 1484–1490 in *DMP1* exon 6 manifested tooth abnormalities at age 3+ years, including enlarged pulp/root canals, thin dentin, and unexpected defects in enamel formation resembling dentinogenesis imperfecta III. In contrast, targeted expression of the 1484–1490del in mice led to a similar but milder tooth phenotype, with limited abnormalities in dentin and normal tooth enamel, and only modest changes in serum FGF23 and Pi levels. Since the recombinant DMP1 activated MAPK signaling, albeit much less potently than wild-type DMP1, it obviously retained some activity. Phenotype differences despite identical *DMP1/Dmp1* mutations suggested that the *DMP1* deletion mutation may trigger unknown changes in adjacent genes. While further studies are required, the phenotypic variability in humans may not be solely from age-dependent penetrance of ARHR, but to mutation-specific coevents.

The mechanisms by which the ARHR mutations modify DMP1 function are variable.[153,158] Transfected HEK293 cells containing wild-type DMP1 expression plasmids show equal expression of DMP1 in cellular lysates and their medium. In contrast and consistent with disordered DMP1 trafficking, HEK293 cells containing the ARHR 1484–1490del and M1V mutants express DMP1 only in the medium and cellular lysates, respectively. Likewise, wild-type and 1484–1490del mutant DMP1 localize to the trans-Golgi network of cells, consistent with their cellular secretion. In contrast, M1V mutant DMP1 did not localize to the trans-Golgi network, but filled the entire cell due to loss of the highly conserved DMP1 signal peptide. Thus, defective DMP1 function in ARHR seems to arise from several mechanisms including gene deletion, absence of protein secretion (M1V mutation), or compromised folding or interference with the overall charge of the mature DMP for secreted proteins (1484–1490del mutation).

Although *DMP1* mutation is rightly viewed as a cause of ARHR, we now know that inactivating mutation of the ectonucleotide pyrophosphatase/phosphodiesterase 1 gene (*ENPP1*) also causes a form of ARHR.[159] In 2010, studies of a Bedouin family revealed classical biochemical and radiographic abnormalities of ARHR, including high-normal to elevated circulating FGF23 levels, but linkage of their disorder to *PHEX, FGF23*, and *DMP1* was excluded. Then, further investigations identified linkage to chromosomal 6q23 with a significant LOD score of 3.45 for D6S262, assuming autosomal recessive

inheritance with 99% penetrance and an incidence of 0.01 or 0.001 for the disease allele in the population. The linkage interval of 7.39 Mb contained 70 genes, including *ENPP1* that contained an inactivating mutation arising from a phenylalanine substitution for the strictly conserved tyrosine in position 901 within the nuclease-like domain. *ENPP1* acts importantly in the generation and disposition of extracellular inorganic pyrophosphate, a potent inhibitor of mineralization. Previously reported inactivating mutations of *ENPP1* caused generalized arterial calcification of infancy (GACI). In GACI, vascular calcification is caused by the paucity of extracellular inorganic pyrophosphate and often proves lethal in infancy. Inactivation of *Enpp1* in mice results in ectopic calcification of joints and ligaments. In some GACI patients, however, a variable and incomplete reciprocal association manifests between aberrant calcification and hypophosphatemic rickets. As hypophosphatemia could theoretically compensate for the excessive mineralization of GACI, it has been assumed that hypophosphatemia reflected a physiological compensation rather than a primary defect. However, the complete dissociation between GACI and ARHR, observed by Levy-Litan et al.[159] in 2010, made a compensatory change unlikely, and strongly suggested that *ENPP1* mutation is a second cause for ARHR. Now, GACI is attributed not only to biallelic *ENPP1* deactivation, but also to biallelic loss-of-function mutations in *ANK* whose protein product acts as a channel for PPi into the extracellular space.[160]

4.3.3 Molecular Genetics and Animal Models

Study of ARHR in humans and animal models has clarified whether the pathogenetic link among the genetic hypophosphatemias is increased circulating FGF23.[161] After the role of FGF23 in the pathogenesis of ADHR and XLH was established, efforts focused on whether increased circulating FGF23 in ARHR and *Dmp1*-null mice caused the hypophosphatemia, diminished $1,25(OH)_2D$ production, and impaired bone mineralization. Although a role of FGF23 in the pathogenesis of ARHR seemed intuitive, the observations that DMP1 localizes to the mineralization front and functions in bone as a nucleator for mineralization within the ECM suggested instead that ARHR rickets and osteomalacia could be mediated directly by the deficient DMP1. Alternatively, the colocalization of PHEX and DMP1 in osteocytes together with the association of DMP1 mutations with increased FGF23 expression, hypophosphatemia, and reduced circulating $1,25(OH)_2D$ levels indicated that excess FGF23 contributed to the ARHR phenotype. The studies of Liu et al.[162] established that control of ECM mineralization by DMP1 is coupled to renal Pi handling and vitamin D metabolism through the DMP1-dependent regulation of FGF23 production by osteocytes. In double knock-out mice ($Dmp1^{-/-}$/$Fgf23^{-/-}$) lacking both *Fgf23* and *Dmp1*, circulating FGF23 levels were as expected undetectable, and the serum Pi and $1,25(OH)_2D$ levels were elevated (as in $Fgf^{-/-}$ mice). This combination transformed the rickets and osteomalacia of $Dmp^{-/-}$ mice to severe growth retardation and focal osteomalacia characteristic of $Fgf23^{-/-}$ mice. Hence, misregulation of FGF23 is principally responsible for the rickets and diffuse osteomalacia in *Dmp1*-null mice and presumably in people with ARHR. *Dmp1* mutation lowers the set-point for Pi and maintains it through regulation of Fgf23 cleavage and expression.[163] It also seems to compromise skeletal muscle functions.[164] DMP1, PHEX, and FGF23 appear to coordinate mineralization and systemic Pi homeostasis, and thereby link the pathogenesis of ARHR, XLH, and ADHR.[165]

However, the precise role of FGF23 in ARHR remains incompletely explained. Restoring serum Pi to control levels in the *Dmp1*-null mouse corrects the mineralization defect at the growth plate, with marked improvement in the bone formation rate, consistent with healing of rickets. However, normalization of serum Pi only improves the osteomalacia; thus the bone phenotype is not completely corrected. Taken together, the data suggest that the rickets of ARHR is due to FGF23-mediated hypophosphatemia, whereas the osteomalacia mostly reflects the defective mineralization resulting from a lack of functional DMP1 in the osteocytes and their microenvironment; an unknown direct effect of FGF23 on bone/OBs; or DMP1-dependent altered transcription of an unknown protein in the OB (see later).

As noted earlier, regulation of *Fgf23* mRNA in *hyp*-mice requires reduction in the 57-kDa C-terminal fragment of DMP1 (Fig. 40.2C). While this abnormality in *hyp*-mice is linked to a decrease in a furin-like protease 7B2 • PC2, in *Dmp1*-null mice and in patients with *DMP1* mutations the limited availability of functional DMP1 reduces the production of the 57-kDa C-terminal fragment, linking the aberrant regulation in *FGF23* mRNA production in XLH and ARHR. Indeed, the *Dmp1*-null phenotype was corrected using a breeding strategy to introduce the Col1a-57 K transgene into *Dmp1*-null mice and generate animals overexpressing it in bone at the mRNA and protein level.[135,163] Hence, the DMP1 57-kDa fragment contains the essential functional domain of DMP1 and regulates *Fgf23* mRNA similarly in ARHR and XLH (see earlier). Consistent with these observations, experimental evidence indicated a dual biological function for DMP1: (1) as a transcriptional signal during early differentiation of OBs, and (2) as an initiator of mineralization during the terminal differentiation of OBs.[164] Different spatial and temporal profiles of DMP1 lead to the different pleiotropic effects. The nuclear localization of DMP1 is osteocyte specific and dependent on binding of the C-terminal NLS3 domain to soluble transport factors, such as α-importin. Once intranuclear, DMP1, or its functional 57-kDa fragment, directly

activates transcriptional pathways, leading to expression of ALP and osteocalcin. It remained unknown whether the DMP1 57-kDa fragment directly activated the FGF23 transcriptional pathway or acted by mediating transcription of FGF2, a known FGF23 regulator in *hyp*-mice.[166] In addition, it was unclear if the DMP1 57-kDa fragment altered the transcriptional pathway of other protein(s), having regulatory effects upon bone mineralization.

4.3.4 Genetic Testing

The clinical presentations of ADHR, XLH, and ARHR are similar. Thus, diagnosing ARHR can depend upon ascertaining its autosomal recessive pattern of inheritance. Consanguinity and/or absence of the disease in the parents supports the diagnosis. Presence of its unique tooth or bone abnormalities also facilitates diagnosis of ARHR. In some cases, absence of consanguinity, insufficient family information, and/or the possibility of mild undiagnosed disease in the mother can preclude a clinical diagnosis. Recently, *DMP1* mutation analysis has become available from commercial laboratories to provide an accurate diagnosis, underpin genetic counseling if requested, and choose the correct therapeutic intervention if ADHR has not already been excluded. ARHR is treated with Pi and $1,25(OH)_2D$ whereas other renal Pi wasting disorders, particularly ADHR, may require less aggressive treatment or therapy with alternative drugs, such as iron. Thus, unambiguous diagnosis of ARHR seems beneficial. Now, *ENPP1* as well as *DMP1* mutation analysis is available in fee-for-service or research laboratories.

4.3.5 Treatment

ARHR pretreatment studies should include: (1) fasting serum assayed for phosphorus, calcium, ALP, creatinine, PTH, 25(OH)D, $1,25(OH)_2D$, and FGF23 together with; (2) simultaneous urine collection(s) to measure calcium, phosphorus, and creatinine excretion and to calculate TRP and TmP/GFR; (3) radiographs of wrists and knees to assess the severity of rickets and elsewhere to document long bone deformities and fractures; and (4) a detailed family history, supported by laboratory investigations, when beneficial. Bone biopsy to document and assess the severity of osteomalacia may be useful under certain circumstances, including poor response to treatment or markedly abnormal skeletal radiographs.

However, few case reports assess therapy for ARHR. Approaches include combination therapy with oral Pi and $1,25(OH)_2D$, as for the management of XLH, yet serum Pi may increase without correction of renal Pi wasting. Nevertheless, resolution of rickets has been noted, as documented in the *Dmp1*-null mouse given increased dietary phosphorus. Intermittent bone pain has diminished with combination therapy.

5 OTHER DISORDERS

Finally, a number of heritable "tubulopathies," such as neuropathic cystinosis (OMIM #219800) and Lowe syndrome (OMIM #309000), and a variety of other entities, are Mendelian disorders associated with hypophosphatemic skeletal disease where the pathogenesis is less well understood.[167] Furthermore, although not heritable, several entities merit mention in this chapter because they are in fact genetic forms of hypophosphatemic rickets/osteomalacia.[167] Here, postzygotic mosaicism involving a number of gene defects represents the etiology and pathogenesis. Included are McCune–Albright syndrome (OMIM #174800) due to activating mutations within a highly specific region of *GNAS1* (Fig. 40.7) or *NRAS* or *KRAS* in the vitamin D-resistant rickets associated with epidermal nevus syndrome (OMIM #163200) (Fig. 40.8).[168] In these disorders too, elevated circulating FGF23 is pathogenic.

FIGURE 40.7 The abdomen and flank of this girl with McCune–Albright syndrome shows a characteristic large "rough-border" café-au-lait spot.

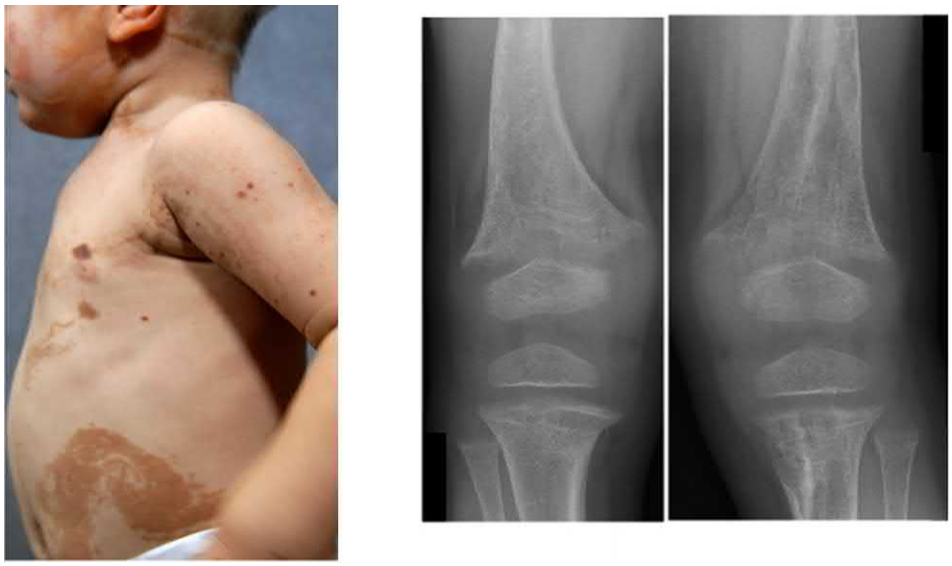

FIGURE 40.8 This 3-year-old boy with an extensive left-side epidermal nevus has hypophosphatemic rickets. Radiographic abnormalities characteristic of the associated skeletal disease, as well as rickets, are seen ipsilaterally in his left knee, whereas only rickets is seen in his right knee.

6 CONCLUSIONS

6.1 Model

ADHR, XLH, and ARHR are heritable disorders that feature hypophosphatemia leading to skeletal disease and arise from mutations in, or dysregulation of, a single gene product in common, FGF23. Osteocyte regulation of Pi homeostasis and bone mineralization is key for the pathophysiology of these heritable disorders that feature rickets and osteomalacia.[169]

1. An *inactivating PHEX mutation* through downstream effects→↑Serum FGF23 [↑FGF23 mRNA and ↓FGF23 proteolysis]→XLH.
2. An *activating FGF23 mutation*→↑Serum FGF23 [↓FGF23 proteolysis]→ADHR.
3. An *inactivating DMP1 mutation* through the direct effects of proteolytic DMP1 fragments→↑Serum FGF23→*ARHR*.

6.2 Predictions

Alterations in FGF23 function are fundamental to this model, but it does not require FGF23 as the sole protein influencing the phenotype of these disorders. The various causal gene mutations will give rise to diseases with a similar phenotype and constellation of complications. The disease severity will, in general, reflect the magnitude of the disturbance in the common intermediate, FGF23. Differences among the entities will actually be minor, likely arising from side pathways, perhaps representing separate functions of the mutated protein beyond the common network.

6.3 Clinical Implications

The investigational human anti-FGF23 monoclonal antibody, burosumab, offers a means to treat the excessive circulating FGF23 in XLH, and will likely be useful for TIO and perhaps MAS and linear nevus syndrome complicated by hypophosphatemia, but it is less certain it will help ADHR or significantly impact ARHR. Hence, understanding the functional network and the different entry points at which FGF23 expression is regulated will increase the number of potential therapeutic targets for these diseases. Perhaps it will be possible to modulate the regulatory pathways and stability mechanisms. Understanding the complex intertwined pathogeneses of these disorders will likely help predict or minimize any side effects arising from genetic or pharmaceutical treatment strategies.

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CHAPTER

41

Renal Fanconi Syndrome, Dent Disease, and Bartter Syndrome

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1 BRIEF CLINICAL DESCRIPTION

Renal Fanconi syndrome (FS) is characterized by a generalized dysfunction of the proximal tubule (PT) of the kidney and a metabolic bone disease.[1–3] A generalized renal PT dysfunction leads to nonselective urinary wasting of amino acids (AAs), glucose, phosphate, uric acid, bicarbonate, and other solutes. The patients develop failure to thrive, polyuria, polydipsia, dehydration, and rickets in children, and osteoporosis and osteomalacia in adults.

2 PATHOPHYSIOLOGY OF THE DISEASE

2.1 General Considerations

The epithelial cells lining the PT reabsorb proteins that have been filtered by the glomerulus, including albumin, low-molecular-weight (LMW) proteins, AAs, glucose, bicarbonate, sodium, chloride, potassium, phosphate, and uric acid. These transport processes are mediated by megalin/cubilin-dependent endocytosis and sodium (Na^+) gradient-dependent transport systems. In FS, endocytosis and/or transport systems mediated by PT cells are disturbed by accumulated substances, immunological injuries, or defective transporters.[4]

Filtered proteins from glomeruli bind to megalin/cubilin in the luminal membrane of PT cells (Fig. 41.1). Cubilin is also interacting with amnionless at the apical membrane of the cells.[5,6] The protein–receptor complex is incorporated into endosomes, before dissociation and recycling back of receptors to the luminal membrane while the reabsorbed proteins are transported to lysosomes for further processing. The progression along the endolysosomal pathway is dependent on vesicular acidification, which itself is due to the function of H^+-ATPase (proton pump) and ClC-5 chloride transport. An abnormal endocytosis pathway may affect the recycling of transport proteins and receptors to the luminal membrane, leading to decreased solute reabsorption.

Reabsorption of filtered solutes, including glucose, phosphate, AAs, and bicarbonate by PT cells is accomplished by transport systems on the apical membrane that are directly or indirectly coupled to Na^+ movement, by energy production and transport from the mitochondria, and by the Na^+, K^+-ATPase at the basolateral membrane. The Na^+, K^+-ATPase lowers intracellular Na^+ concentration and provides the electrochemical gradient that allows Na^+-coupled solute entry into the cell. Primary or secondary mitochondrial dysfunction disturbs ATP production and impairs the operation of Na^+,K^+-ATPase and other membrane carriers that are involved with solute reabsorption in the PT. A defect in energy generation in the PT cells thus produces multiple transport abnormalities that characterize the FS.

2.2 Metabolic Bone Disease: Rickets and Osteomalacia

Growth retardation is a common feature of FS in children.[8] Malnutrition, hypokalemia, hypophosphatemia, and metabolic acidosis lead to growth retardation in patients with FS. Hypokalemia reduces circulating levels of growth hormone (GH) and insulin-like growth factor I (IGF-I).[9] It also decreases appetite, leading to malnutrition and extracellular volume contraction. Metabolic

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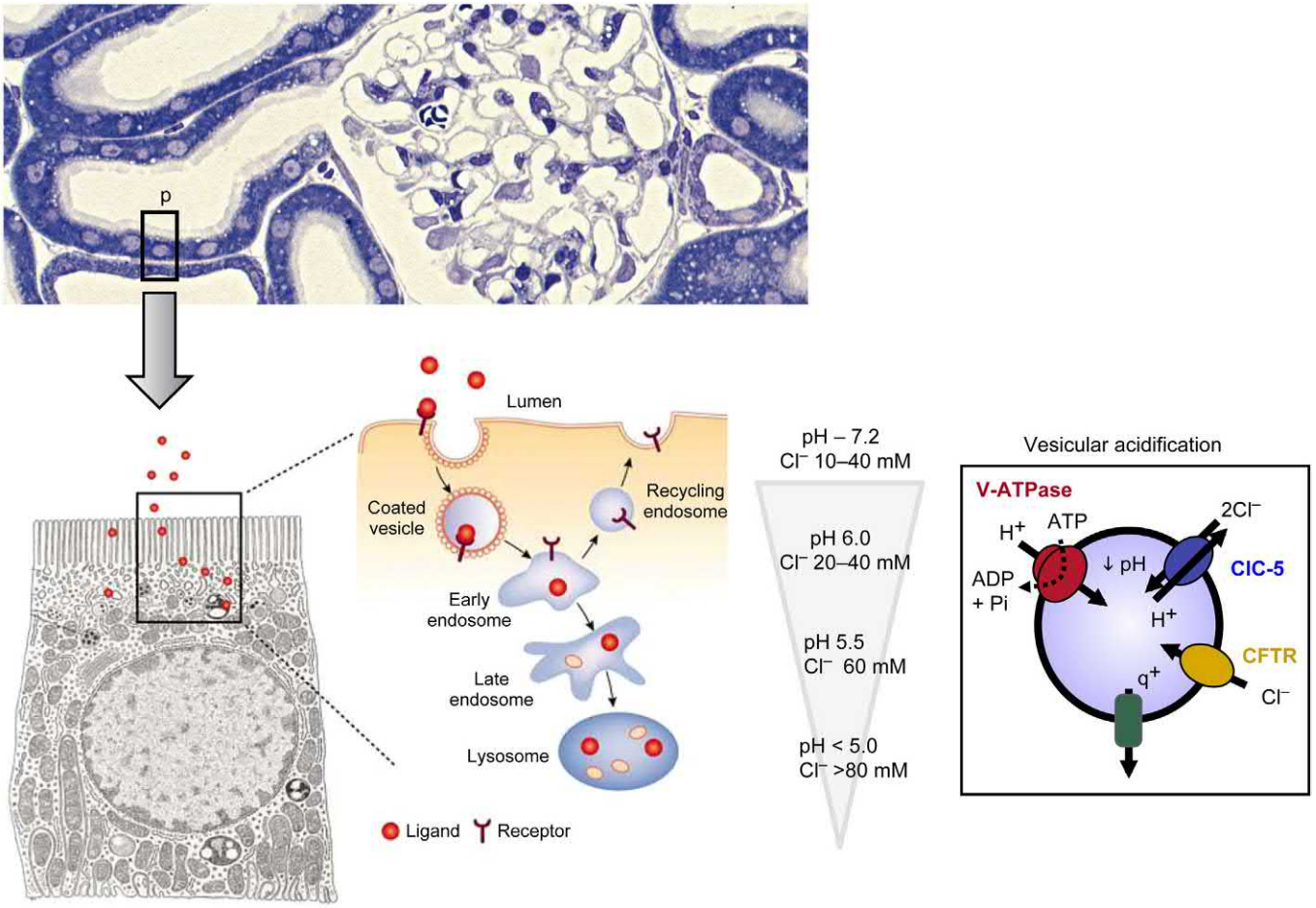

FIGURE 41.1 Receptor-mediated endocytosis and role of ClC-5 in endosomes of proximal tubule (PT) cells. The epithelial cells lining the PT segments (p) of the kidney are characterized by their capacity to reabsorb low-molecular-weight (LMW) proteins (*gray dots*) that are ultrafiltered by the glomerulus. The apical, receptor-mediated endocytic pathway involves coated pits and coated vesicles, followed by early endosomes that form recycling endosomes or mature to late endosomes and lysosomes. Progressive vesicular acidification (from pH 7.2 in the cytosol down to pH <5.0 in lysosomes) and, possibly, changes in vesicular chloride concentrations (from 20 to 40 mM in early endosomes to >80 mM in lysosomes) are necessary for progression along the endocytic apparatus, dissociation of the ligand–receptor complex in lysosomes, and recycling of receptors to the apical membrane. Vesicular acidification is achieved by ATP-driven transport of cytosolic H^+ through the vacuolar proton pump (V-ATPase). In apical endosomes, the ClC-5 Cl^-/H^+ exchanger provides a countercurrent for the proton pump, which facilitates vesicular acidification. Other chloride transporters like the CFTR channel may also play a role. Mutations in ClC-5 impair receptor-mediated endocytosis and progression along the endolysosomal pathway, altering the transport processes mediated by PT cells and causing renal Fanconi syndrome (FS). *Source: Modified from Christensen EI, Birn H. Megalin and cubilin: multifunctional endocytic receptors.* Nat Rev Mol Cell Biol *2002;***3***:256–66.*[7]

acidosis inhibits GH secretion, the expression of IGF-I, and GH receptor.

Rickets and osteomalacia in patients with FS are caused by the urinary wasting of phosphate, as well as by impaired 1α-hydroxylation of 25-hydroxy vitamin D3 by PT cells. In addition, many patients with FS due to Dent disease also have hypercalciuria.

Rickets manifests as bowing deformity of the lower limbs, distal femur, the ulna, and the radius. Osteomalacia presents bone and joint pains in the hips, shoulders, and trunk, and difficulty of walking due to multiple bone fractures. Hypomineralization of dentin structure and immature formation of craniofacial bones are seen in patients with FS.

Metabolic acidosis impairs the conversion of 25-hydroxyl vitamin D3 to 1,25-dihydroxy vitamin D3. Parathyroid hormone (PTH) is filtered from glomeruli, reabsorbed and degraded along the endolysosomal pathway in the PT.[10] Megalin/cubilin and endosomal trafficking in the PT is impaired in FS. Failure to clear luminal PTH stimulates 1-α-hydroxylase but inappropriately low 1,25-dihydroxy vitamin D3 levels are seen in FS.[11] This is produced by the defective megalin/cubilin dependent endocytosis that leads to urinary loss of LMW vitamin D–binding protein. As internalization of 25-hydroxy vitamin D3—LMW binding protein complex is normally mediated by megalin/cubilin dependent endocytosis in the PT.

2.3 Polyuria, Polydipsia, and Dehydration

Polyuria, polydipsia, and dehydration are seen in patients with FS. Polyuria is secondary to the osmotic diuresis from the excessive urinary solute losses and urine concentration defect in the collecting ducts due to chronic hypokalemia. Recurrent acute fever due to dehydration is a frequent manifestation in infants with FS.

2.4 Urinary Bicarbonate Loss and Acidemia

More than 85% of filtered load of bicarbonate (HCO_3^-) is reabsorbed by PT cells. This is accomplished by the coordinated function of luminal membrane Na^+/H^+ exchanger, luminal membrane carbonic anhydrase isoforms IV and XIV, and basolateral membrane Na^+/HCO_3^- cotransporter. Hyperchloremic metabolic acidosis is a common feature of FS resulting from defective bicarbonate reabsorption by PT cells. More than 30% of filtered load of HCO_3^- is not reabsorbed in FS and plasma HCO_3^- levels decrease to 12–18 mEq/L.

2.5 Glucosuria

The filtered load of glucose (MW = 180 D) is almost completely reabsorbed by a sodium-coupled active transporter (SGLT1/2) in the brush border membrane of the PT. Glucose reabsorption involves coupled transporters at the luminal and basolateral membranes of the PTs.[12] The driving force for glucose reabsorption is provided by Na^+, K^+-ATPase in the basolateral membrane. Between 0.5 and 20 g of glucose are lost in the urine in patients with FS in a day.

2.6 Phosphaturia and Hypophosphatemia

The patients manifest phosphaturia and hypophosphatemia. Two sodium phosphate cotransporters, NaPi-IIa and NaPi-IIc, are expressed on the luminal membrane of PT cells and reabsorb 70%–80% of filtered phosphate.[13] Defective NaPi-IIa causes FS. PTH and vitamin D3 affect the phosphate handling in the kidney. Failure to clear luminal PTH results in increased activation of luminal PTH receptors resulting in internalization of NaPi-IIa and NaPi-IIb, and thus phosphaturia.

2.7 Urinary Sodium Loss and Secondary Potassium Loss

Approximately two-thirds of filtered load of Na^+ is reabsorbed in the PTs. This renal Na^+ reabsorption decreases in patients with FS, causing hypotension and dehydration. Increased Na^+ delivery to the distal tubules and activation of renin–angiotensin system secondary to hypovolemia lead to K^+ wasting in the distal tubules.

2.8 Uricosuria and Hypouricemia

Uric acid (MW = 126 D) is the end product of purine metabolism in humans. It is filtered from the glomeruli and 90%–95% of uric acid is reabsorbed in the PTs. Uricosuria is often present in FS, leading to secondary hypouricemia (<2 mg/dL). Uric acid–anion transporter (URAT1) and glucose transporter 9 (GLUT9) regulate serum uric acid levels.[14] The residual apical uptake of uric acid is mediated by organic anion transporter (OAT) 4 and OAT10.[15,16] URAT 1 is located on luminal membrane of the PT cells. GLUT9 has two isoforms; the long isoform (GLUT9L) is expressed in basolateral membrane and the short isoform (GLUT9S) is expressed in luminal membrane of PT cells.[17,18] URAT1 and GLUT9S reabsorb uric acid from the lumen to PT cells and GLUT9L transports uric acid from PT cells to the tissue. These uric acid transporters are disturbed in FS. Uric acid is an important antioxidant in humans: over half the antioxidant capacity of blood plasma comes from uric acid.[19]

2.9 Generalized Aminoaciduria

AAs are filtered from glomeruli and 95%–99% of filtered AAs are reabsorbed in the PTs. Fractional excretion of AA is usually less than 3% in normal controls except for neonate or premature babies. However, only histidine has a fractional excretion of 5%. The condition in which the fractional excretion of AA is more than 5% is termed aminoaciduria. Every AA is highly excreted in patients with FS (generalized aminoaciduria).[20]

2.10 Low-Molecular-Weight Proteinuria

LMW proteinuria, including proteins with a mass equal or lower than albumin (60 kD), is seen in patients with FS, reflecting the defective megalin/cubilin dependent endocytosis.[21] Filtered load of 99mTc-DMSA (technetium-99m-dimercaptosuccinic acid) is reabsorbed by PT segments. Renal uptake of 99mTc-DMSA decreased in patients with LMW proteinuria.[22]

2.11 Decreased Urinary Excretion of Osteopontin and Uromodulin

Osteopontin is excreted into the urine from renal tubular cells in cases of stress. It has a role in the remodeling of extracellular matrix and inhibition of apoptosis. It is also a protective factor against nephrocalcinosis. Uromodulin (Tamm–Horsfall protein) is excreted by renal tubular cells and plays a major role in combating urinary tract infections. Urinary excretion of both proteins is reduced in the patients with FS.[23]

3 ETIOLOGIES

The causes of FS are shown in Table 41.1.

3.1 Idiopathic Fanconi Syndrome

Although an increasing number of genetic causes for the disorders leading to FS have been identified, there exist patients with idiopathic FS.

3.2 Hereditary Fanconi Syndrome

3.2.1 Phosphate Transporter NaPi-IIa Deficiency

This disorder is an autosomal recessive proximal tubulopathy associated with severe renal phosphate wasting, hypophosphatemic rickets, osteopenia, and renal insufficiency due to the defective sodium phosphate cotransporter NaPi-IIa gene (*SLC34A1*).[24] The mutations in the *SLC34A1* gene cause autosomal recessive FS, hypophosphatemic rickets, hypercalciuria, and elevated serum 1,25-dihydroxyvitamin D3 levels.[25] Treatment with oral phosphate supplements results in normalization of serum phosphate and 1,25-dihydroxyvitamin D3 levels, reversal of hypercalciuria, and radiologic resolution of rickets, but had no effect on the proximal tubulopathy.[26]

3.2.2 Mitochondriopathies

Genetic defects of one or several polypeptide enzyme complexes of the oxidative phosphorylation system in the mitochondrial DNA or nuclear DNA which encodes structural or functional mitochondrial proteins give rise to mitochondriopathies.

Mitochondriopathies are multisystemic disorders that present at any age. The extreme heterogeneity makes the extent and manifestations of disease presentation make unpredictable.[27] With the progress of the disease over time, an increasing number of organs may be involved.

Many patients manifest FS by the age of 2 years. Histological analysis reveals tubular dilatations, tubular atrophy, and cytoplasmic vacuolization of the tubules. Bizarre giant mitochondria are frequently observed.

TABLE 41.1 Causes of Renal FS

1. Idiopathic
   a. Autosomal dominant
   b. Autosomal recessive
   c. X-linked
2. Hereditary
   a. Phosphate transporter NaPi-IIa deficiency
   b. Mitochondriopathies
   c. Lowe syndrome
   d. Cystinosis
   e. Galactosemia
   f. Hereditary fructose intolerance
   g. Glycogen storage disease type I (von Gierke disease)
   h. Fanconi-Bickel syndrome
   i. Tyrosinemia type I
   j. Wilson disease
   k. Maturity-onset diabetes of the young, types 1 (HNF4α), 3 (HNF1α), and 5 (HNF1β)
   l. Lysinuric protein intolerance
   m. Microvillous inclusion disease
   n. Dent disease
3. Acquired
   a. Monoclonal gammopathies
   b. Sjögren syndrome
   c. Renal transplantation
   d. Acute tubulointerstitial nephritis with uveitis syndrome
   e. Autoimmune interstitial nephritis and membranous nephropathy
   f. Primary biliary cirrhosis
   g. Renal hemosiderosis
4. Exogenous substances
   a. Drugs: aminoglycosides, salicylate, valproic acid, Chinese herbs, ifosfamide, cisplatin, imanitib mesylate, adefovir, cidofovir, tenofovir, zoledronic acid, deferasirox
   b. Chemical compounds: paraquat, bismus, methyl-3-chromone, 6-mercaptopurine, toluene
   c. Heavy metals: lead, cadmium, mercury, chromium, platinum
   d. Honeybee stings: mellitin

3.2.3 Lowe Syndrome

The oculocerebrorenal syndrome of Lowe (OCRL) is an X-linked disorder that is characterized by ocular manifestations (particularly congenital cataracts), central nervous system abnormalities, and FS.[28] Central nervous system abnormalities include infantile hypotonia, areflexia, and mental retardation. FS is a major clinical feature and occurs in the first year of life, but the severity and age of onset vary. The causative gene (*OCRL*) encodes a 105 kD Golgi protein with phosphoinositol 4,5-bisphosphate phosphatase (PIP2 5-phosphatase) activity. PIP2 5-phosphatase is mainly a lipid phosphatase that controls cellular levels of phosphatidylinositol 4,5-bisphosphate (PIP_2), and is involved in the inositol phosphatase-signaling pathway.[29] Deficiency of PIP2 5-phosphatase leads to accumulation of PIP2 in lysosomal membrane. PIP2 is involved in signal transduction, vesicle trafficking, and actin polymerization.

3.2.4 Cystinosis

Cystinosis is an autosomal recessive lysosomal storage disorder characterized by a multisystemic accumulation of cystine. Cystinosis is the most common familial form of the FS in Western countries. Other organs frequently affected include the cornea and thyroid, causing painful photophobia and hypothyroidism, respectively. The most severe form, infantile cystinosis, manifests FS between 6 and 12 months of age. Renal function is normal at presentation. However, subsequent glomerular impairment leads to end stage renal failure by 10 years of age without treatment. Cystinosis is caused by mutations of the *CNTS* gene encoding cystinosin, a lysosomal transport protein.[30] PT cells accumulate intracellular cystine and develop apoptosis. Oxidative stress and altered redox status are observed in cystinosis PT cells. This produces the atubular glomeruli in end-stage cystinotic kidney tissue. Morphologically abnormal pattern of mitochondrial autophagy with a high number of autophagic vacuoles and fewer mitochondria is seen in PT cells.[31] Recent studies have demonstrated that the functional loss of cystinosin in PT cells triggers aberrations of the endolysosomal compartment, transport defects, and an abnormal transcription program in the early stage of nephropathic cystinosis, explaining the early transport defects and the FS.[32]

3.2.5 Galactosemia

Galactosemia is an autosomal recessive disease of galactose metabolism. The most frequent form is classic galactosemia due to the defective galactose-1-phosphate uridyl-transferase (GALT) gene (*GALT1*).[33] GALT catalyzes the reaction of galactose-1-phosphate (gal-1-p) plus uridine diphosphate glucose to uridine diphosphate galactose plus glucose-phosphate. Uridine diphophate galactose can be further metabolized to either glucose or CO_2 and H_2O via glycolysis. Milk is a major source of galactose. Accumulated gal-1-p and exposure to galactose lead to acute deterioration of multiple organs. Affected infant patients manifest vomiting, diarrhea, failure to thrive, developmental delay, liver dysfunction, coagulopathy, FS, cerebral edema, vitreous hemorrhage, and *Escherichia coli* sepsis.

3.2.6 Hereditary Fructose Intolerance

Hereditary fructose intolerance is caused by a deficiency of aldolase B.[34] Aldolase B catalyzes the specific and reversible cleavage of fructose-1,6-bisphophate (FBP) and fructose-1-phosphate (F1P) into dihydroxyacetone phosphate and D-glyceraldehyde-3-phophate, or D-glyceraldehyde. Aldolase B is equally active with FBP and F1P, whereas aldolase A and C, the other two vertebrate isozymes, are more active with FBP than with F1P. Aldolase B is encoded by the *ALDOB* gene. Aldolase B coexists abundantly in endocytosis zones of the PT cells with H^+-ATPase. Nonfunctional aldolase B impairs the coupling of H^+-ATPase to glycolysis and endosomal acidification that leads to FS. Affected individuals manifest symptomatic hypoglycemia, vomiting, and life-threatening episodes shortly after the intake of fructose or related sugars. Prolonged ingestion leads to failure to thrive, hepatomegaly, jaundice, hepatic cirrhosis, nephrocalcinosis, convulsions, coma, and death from severe liver and kidney failure.

3.2.7 Glycogen Storage Disease Type I (von Gierke Disease)

There are two major subtypes of glycogen storage disease type I (GSD-I). Type Ia (GSD-Ia, von Gierke disease) is the most frequent, caused by a deficiency in glucose-6-phosphatase-alpha (G6Pase-α) that catalyzes the hydrolysis of glucose-6-phosphate (G6P) to glucose and phosphate in the terminal step of gluconeogenesis and glycogenolysis.[35] G6Pase-α is a hydrophobic endoplasmic reticulum–associated transmembrane protein. Type Ib (GSD-Ib) is caused by a deficiency of microsomal glucose-6-phosphatase transporter (G6PT). G6PT translocates G6P from cytoplasm to the lumen of the endoplasmic reticulum.

Patients with GSD-Ia and GSD-Ib manifest life-threatening hypoglycemia, hepatomegaly, nephromegaly, hypercholesterolemia, hypertriglyceridemia, hyperuricemia, lactic acidemia, neutrophilia, and growth retardation. Infants with GSD-Ia typically present with seizures and hepatomegaly at 6–8 months of age. Approximately 75% of adolescent and adult patients develop hepatocellular adenoma.

3.2.8 Fanconi–Bickel Syndrome

Fanconi–Bickel syndrome (FBS) is characterized by failure to thrive, "doll-like" face, hepatomegaly, nephromegaly, and severe rickets. Patients with FBS manifest

glycogen accumulation in hepatocytes and PT cells, fasting hypoglycemia, galactose intolerance, and FS.[36] Some patients manifest cataracts in neonatal period. The overall prognosis of FBS is considered as favorable. However, some patients manifest neonatal diabetes mellitus and galactosemia and die of hepatic failure during infancy. FBS is caused by mutations in the *SLC2A2* gene coding for the glucose transporter GLUT2 that is expressed in liver, kidney, intestine, and pancreatic islet cells.[37]

3.2.9 Tyrosinemia Type I

Hereditary tyrosinemia type I (TI) is an autosomal recessive disorder of an AA metabolism due to the defect in the fumarylacetoacetate hydrolase (*FAH*) gene.[38] Patients with TI display a variety of clinical symptoms, such as liver damage from infancy that advances to cirrhosis, reduced coagulation factors, hypoglycemia, high plasma methionine, phenylalanine, and aminolevulinic acid, high risk of hepatocellular carcinoma, renal dysfunction and FS.

3.2.10 Wilson Disease

Wilson disease (WD) is an autosomal recessive disorder of copper (Cu) metabolism. Biliary excretion of Cu and incorporation into ceruloplasmin is impaired, leading to liver damage, neuronal degeneration, and impairment of other organs from accumulation of Cu in the patients. WD is caused by a mutation in the *ATP7B* gene that encodes a P-type Cu transporting ATPase-β polypeptide enzyme (ATP7B).[39] Untreated patients with WD manifest FS.

3.2.11 Maturity-Onset Diabetes of the Young (MODY) Types 1, 3, and 5

Hepatocyte nuclear factor 4α (HNF4α) is a nuclear transcription factor that binds DNA as a homodimer. HNF4α controls the expression of several genes, including HNF1α, a transcription factor that regulates the expression of several genes in liver, kidney, and intestine. Autosomal dominant mutations in the *HNF4A* gene that encodes HNF4α usually lead to neonatal hyperinsulinism and young onset diabetes mellitus (maturity-onset diabetes of the young type 1, MODY1).[40] Patients harboring the R76W mutation of *HNF4A* manifest autosomal dominant FS in addition to neonatal hyperinsulinism, MODY, and macrosomia.[41] Recently, Terryn et al. showed that HNF1α directly regulates the transcription of the megalin and cubilin receptors in vivo and that mice knockout (KO) for HNF1α present LMW proteinuria and a major endocytic defect due to decreased expression of megalin/cubilin receptors. Furthermore, LMW proteinuria was consistently detected in individuals with *HNF1A* mutations (MODY3) compared with healthy controls and patients with non-MODY3 diabetes mellitus.[42]

MODY5 is one of hereditary diabetes mellitus that is dominantly inherited due to the mutations in HNF1β gene (*HNF1B*). The diabetes mellitus associated with this disease appears in children or in young adult. The patients manifest bilateral renal cystic hypodysplasia during childhood. In contrast, renal involvement in adulthood is extremely heterogeneous, with a tubulointerstitial profile at presentation and slowly progressive renal decline throughout adulthood. Adult patients may present renal cysts, a solitary kidney, or tubular manifestations, including hypokalemia and hypomagnesemia. They may also present with renal FS and progress to end-stage renal failure.[43,44]

3.2.12 Microvillous Inclusion Disease

Microvillous inclusion disease (MVID) is one of the most severe congenital intestinal malabsorption disorder that presents with intractable secretory diarrhea within a few days or weeks of life, leading to life-threatening malnutrition and dehydration. Affected infants manifest developmental delay, liver and kidney damage, and rickets. Some affected individuals develop cholestasis and liver cirrhosis. In individuals with MVID, lifelong nutritional support is needed and given through total parenteral nutrition. MVID is associated with brush border atrophy and is caused by mutations in the *MYO5B* gene coding for myosin Vb, an actin-based motor protein that binds to specific small GTPase rab proteins on recycling endosomes, and transports these along with actin filaments to the apical plasma membrane.[45] Loss of expression or function of the myosin Vb protein impairs apical brush border membrane formation and cell polarity. As a result, enterocytes cannot properly form microvilli, which absorb nutrients and fluids from food as it passes through the intestine. Loss of microvilli in PT cells in the patients can result in reabsorption defect and FS that is also observed in patients with lysinuric protein intolerance (LPI).[46] In addition, defects in apical protein trafficking, in particular the recycling of brush border proteins via apical endosomes in PT cells can lead to FS.

3.2.13 Lysinuric Protein Intolerance

LPI is an autosomal recessive metabolic disorder affecting AA transport.[47] Patients with LPI develop vomiting and diarrhea after weaning, resulting in failure to thrive, poor appetite, growth retardation, enlarged liver and spleen, prominent osteoporosis and osteopenia, delayed bone age. Due to the low protein concentration in human milk, they are usually symptom-free during infancy. Forced feeding of protein leads to convulsions and coma. Mental development is normal if prolonged episode of hyperammonemia can be avoided. Urinary excretion of cationic AAs (ornithine,

arginine, and lysine) is increased and their plasma concentrations are low. Deficiency of arginine and ornithine restricts the function of the urea cycle and leads to hyperammonemia after protein-rich meals. LPI is caused by the mutations of *SLC7A7* gene, encoding a permease-related protein.[48] Ultrastructural analysis of PT cells showed vacuolization and sloughing of the apical brush border. Loss of microvilli in PT cells in these patients can result in reabsorption defect and FS.[45] The intratubular accumulation of lysine also can induce PT dysfunction.[49]

3.3 Acquired Fanconi Syndrome

Immunological or hematological disorders are associated with dysproteinuria leading to FS. Sjögren syndrome is an autoimmune connective tissue disorder that affects exocrine glands leading to FS.[50] Corticosteroid or/and immunosuppressant therapy can improve the prognosis. Patients with lymphoma, especially acute T cell leukemia/lymphoma that is related to human T cell lymphotrophic virus 1 (HTLV1) infection, manifest FS.[51] A kidney biopsy reveals intense PT infiltration by lymphoma cells. Signs and symptoms regress following the successful treatment of lymphoma. Monoclonal gammopathies are frequently complicated by kidney lesions that increase the disease morbidity and mortality. In particular, abnormal immunoglobulin–free kappa light chains (κLCs) may accumulate within epithelial cells, causing PT dysfunction and renal FS. Recent studies have shown that specific κLCs accumulate within lysosomes, altering lysosome dynamics, and proteolytic function, causing dedifferentiation and loss of reabsorptive capacity of PT cells.[52]

Acute tubulointerstitial nephritis with ileitis (TINU) syndrome is an immunological disease that leads to tubulointerstitial nephritis and bilateral anterior uveitis. Patients with TINU syndrome manifest asthenia, malaise, weight loss, nocturia, thirst, ocular pain, and blurred vision. Patients also manifest incomplete or complete FS.[53] Corticosteroid therapy can improve renal and ocular manifestations.

Autoimmune interstitial nephritis and membranous nephropathy can present as FS and proteinuria. Renal biopsy revealed interstitial nephritis with lymphocytic infiltration and fibrosis, and membranous nephropathy. Linear staining of IgG along the glomerular capillaries and the tubular basement membrane are also detected.

Primary biliary cirrhosis is a chronic cholestatic liver disease. The patients have serum antimitochondrial antibody-M2 that inhibits pyruvate dehydrogenase and α-ketoglutarate dehydrogenase resulting in tubulointerstitial nephritis and FS.

Renal hemosiderosis secondary to both chronic repetitive hemolytic episodes and transfusion-related iron overload in patients with paroxysmal nocturnal hemoglobinuria can lead to FS. Deteriorating kidney function and persistent FS are accompanied by a progressive increase in serum ferritin.[54]

3.4 Exogenous Factors

3.4.1 Drugs

Numerous drugs and herbs are implicated in the pathogenesis of FS. They include aminoglycosides, salicylate, valproic acid, and Chinese herbs. Aminoglycosides reduce glucose reabsorption in kidney tissue by reducing Na^+-dependent glucose transporter. Covalent binding of salicylate or its metabolites to mitochondria in PT cells alters their function. Valproic acid produces the defects of mitochondrial respiratory chain and lysosomal enzyme activity in the PT cells. Chinese herbs containing aristolochic acids cause proximal tubular injury.

The nephrotoxicity of cancer chemotherapy agents is dose dependent and often irreversible. Chloroacetaldehyde, one of the main metabolites of ifosfamide, inhibits endocytosis in PT cells. Cisplatin reduces glucose reabsorption in kidney tissue by reducing Na^+-dependent glucose transporter. It also inhibits various AA transporters in PT cells. Imanitib mesylate is a specific tyrosine kinase inhibitor that is the first line therapy for patients with chronic myeloid leukemia. This agent can induce partial FS with mild renal failure.

Nucleotide reverse transcriptase inhibitors, such as antihuman immunodeficiency virus and antichronic hepatitis B virus agents, including adefovir, cidofovir, and tenofovir induce FS. Tenofovir is now the most prescribed antiretroviral drug. These nucleotide reverse transcriptase inhibitors are accumulated in PT cells leading to mitochondrial damage.[55]

Zoledronic acid is a bisphosphonate. It is used to prevent skeletal fractures in patients with cancers, such as multiple myeloma and prostate cancer, as well as for treating osteoporosis. It can also be used to treat hypercalcemia of malignancy and is helpful for treating pain from bone metastases. It can produce FS when frequently administered.[56]

Deferasirox is a widely used oral iron chelator for the patients with iron overload due to chronic transfusion therapy for diseases, such as β-thalassemia and sickle cell disease. Deferasirox can produce FS.[57]

3.4.2 Chemical Compounds

Paraquat and colloidal bismuth cause FS. Treatment with the chelating agent sodium-2,3-dimercapto-1-propranesulfonate in combination with hemodialysis is highly effective in reducing the serum bismuth level.

Methyl-3-chromone, 6-mercaptopurine, and toluene can also lead to FS.

3.4.3 *Heavy Metals*

Heavy metals, including lead, cadmium, mercury, chromium, and platinum are a major environmental and occupational hazard. They are very toxic for the PT and cause FS.[58] Important nonindustrial sources of exposure are cigarette smoking and food from cadmium-contaminated soil and water. Cadmium produces free radicals that alter mitochondrial activity or induce mitochondrial gene alterations in the PTs.[58]

3.4.4 *Honeybee Stings*

Honeybee stings have been associated with FS. The renal biopsy revealed dense lymphocytic interstitial infiltration. Acute tubulointerstitial nephritis can be a cause of FS. Another possible pathogenesis of FS is mellitin, a component of bee venom, which inhibits apical transporters of PTs.[59]

4 DIAGNOSTIC ASPECTS

FS is characterized by a generalized dysfunction of the renal PT and a metabolic bone disease. The metabolic bone disease component is not always present. When a generalized renal PT dysfunction leading to unselective urinary wasting of AAs, glucose, phosphate, uric acid, bicarbonate, and other solutes is found, the diagnosis of complete FS is made. When one or two urinary wasting described above is lacking, the diagnosis of partial FS is made. Once the diagnosis of FS is made, the original disease or agents underlying the FS should be identified.

5 GENETIC COUNSELING

Various congenital tubulopathies and inborn errors of metabolism are causing FS (Table 41.1). Genetic counseling is necessary when a genetic cause of FS has been suspected or demonstrated.

6 TREATMENT

6.1 Specific Treatments

Identification of the underlying cause for FS is a first step and is critical to direct specific treatments. Specific treatments for the diseases that lead to hereditary renal FS are shown in Table 41.2. Discontinuation of exogenous factors, such as drugs, foods, chemical compounds, and heavy metals that leads to FS is critical. Immunosuppressive drugs are used for immunologically induced disorders.

6.2 Correction of Fluid and Electrolyte Disturbances

When specific measures do not exist, therapy is directed at the biochemical abnormalities secondary to renal solute and fluid losses and the metabolic bone diseases. Proximal renal tubular acidosis requires large amount of alkali (2–15 mEq/kg of body weight a day) divided into four to six daily doses. 1–3 mg/kg of body weight a day of hydrochlorothiazide can reduce the dose of alkali by preventing the volume expansion. Administration of potassium salt of citrate, bicarbonate, or acetate fulfils the dual purpose of treating acidosis and preventing hypokalemia. Sodium wasting and dehydration are treated with combination of sodium bicarbonate, citrate, and chloride.

6.3 Treatments of Metabolic Bone Diseases and Growth Failure

Oral phosphate supplements in combination with vitamin D3 are the mainstay of treatments for rickets and osteomalacia. 1–3 g of phosphate is necessary as neutral phosphate (the mixture of sodium phosphate dibasic 1.94 g and potassium phosphate monobasic 0.34 g contains 0.5 g of phosphate) divided into four to six daily doses. Parenteral phosphate supplementation is generally reserved for patients with life-threatening hypophosphatemia (serum phosphate < 2.0 mg/dL). Intravenous phosphate (0.16 mmol/kg) is administered at a rate of 1 mmol/h to 3 mmol/h until level of 2 mg/dL is attained. Supplementation of 1,25-dihydroxy vitamin D3 or dihydrotachysterol is effective to treat or prevent rickets and osteomalacia. Vitamin D3 therapy improves the hypophosphatemia and reduces the risk of hyperparathyroidism. An adequate amount of physical activity, as well as appropriate diet with calcium, phosphate, and vitamin D3, is necessary to prevent bone deformations, nontraumatic fractures, deterioration of motor development, and disability. Pamidronic acid (nitrogen containing bisphosphonate) and GH therapy improved tubular reabsorption of phosphate and bone mineral density.[60]

Growth failure is a major complication in children with FS. Despite correction of electrolyte abnormalities, some patients manifest severe growth retardation, especially those with cystinosis and FBS. Earlier diagnosis and efficient correction of acidosis and electrolyte balances by supportive therapy can improve growth and final height in patients with FS. However, supportive therapy is frequently unable to prevent further loss of

TABLE 41.2 Specific Therapies for Inherited Renal FS

Diseases that lead to hereditary renal FS	Specific therapies
Mitochondropathies	Drugs that interfere with the respiratory chain, such as valproate and barbiturates, or that inhibit mitochondrial protein synthesis, such as tetracyclines and chloramphenicol should be avoided in these patients.
Cystinosis	Early diagnosis and oral cysteamine, a cystine-depleting agent, can delay the progression of end stage renal failure and other organ involvement. Cysteamine increases total glutathione and restores glutathione redox status as antioxidant. Oral cysteamine therapy given at doses of 60–90 mg/kg of body weight (or between 1.3 and 1.95 g/m^2) a day divided every 6 h generally achieves approximately 90% depletion of cellular cystine, as measured in circulating leukocytes (<1.0 nmol half-cystine/mg protein). The dosage recommended for adults is 500 mg every 6 h. On the basis of its beneficial effects in maintaining thyroid function and depleting muscle of cystine, oral cysteamine therapy should continue in patients after renal transplantation to help preserve other organs. Administration of 0.55% cysteamine eye-drops, given 6–12 times a day, can dissolve corneal cystine crystals and reduce visual symptoms.
Galactosemia	Treatment is elimination of galactose from the diet. Acute symptoms and signs resolve within a few days after starting the diet therapy. However, developmental delay, speech disturbance, ovarian dysfunction, and growth retardation are common outcomes.
Hereditary fructose intolerance	Strict avoidance of foods or drugs containing fructose, sucrose, and sorbitol is the predominant treatment.
Glycogen storage disease	The treatment goal is to maintain normoglycemia to avoid metabolic complications and lactic acidosis. Normoglycemia is accomplished at night with nasogastric feeding of glucose or with orally administered uncooked cornstarch. A single dose (1.75–2.5 g/kg of body weight) of uncooked cornstarch maintains serum glucose level higher than 3.9 mmol/L for 7 h in most young adults.
Fanconi-Bickel syndrome	Frequent feeding including night-time supplementation is necessary to prevent ketosis. Uncooked cornstarch lessens hypoglycemia and improves growth. Galactose-free milk is also used for infant patients.
Tyrosinemia type I	Treatment with a low-phenylalanine and low-tyrosine diet dramatically improves the renal tubular dysfunction. However, this treatment cannot necessarily improve the hepatic involvement. There is a risk of inducing deficiencies of phenylalanine or tyrosine. The formation of toxic/carcinogenic metabolite (fuamrylacetoacetae) is prevented by 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC). NTBC is used for the patients during the first 6 months of life in addition to a diet low in tyrosine and phenylalanine. NTPC improves the vital prognosis and quality of life in the patients. However, some patients with NTBC treatment develop hepatocellular carcinoma. Liver transplantation leads to rapid correction of FS.
Wilson disease	Patients should avoid Cu-rich foods, including chocolate, nuts, shellfish, mushrooms, and liver. Zinc acetate or zinc sulfate (3–5 mg/kg of body weight a day in three divided doses before meals) has been used successfully used in asymptomatic or presymptomatic affected individuals with WD, and is equally as effective as D-penicillamine (20 mg/kg of body weight a day) in patients with neurological disease. Zinc acetate or sulfate induces intestinal metallothionein and helps in preventing Cu absorption from the gut. D-penicillamine therapy was the most commonly used chelating agent. However, use of D-penicillamine has been questioned, because of reported side effects. Trientine is another effective chelating agent (750–2000 mg a day for adults). Liver transplantation is effective for the patients with progressive live failure or acute liver failure. Liver transplantation is also indicated for patients in whom medical therapy is ineffective.

relative height in patients with FS, especially those with cystinosis. The administration of GH is required in patients with cystinosis or other disorders.

7 DENT DISEASE

7.1 Clinical Description

7.1.1 Dent Disease 1

Dent disease [(Online Mendelian Inheritance in Man (OMIM) #300009] refers to a heterogeneous group of X-linked disorders that have previously been reported as X-linked recessive nephrolithiasis, X-linked hypercalciuric hypophosphatemic rickets, or idiopathic LMW proteinuria with hypercalciuria and nephrocalcinosis.[61–66] The disease is characterized by manifestations of PT dysfunction associated with hypercalciuria, nephrolithiasis, nephrocalcinosis, and progressive renal failure.[67] Dent disease is a rare disorder, with approximately 350 families reported to date.[68–70]

LMW proteinuria is the most consistent manifestation of Dent disease, detected in almost all affected males and obligate female carriers. There is considerable inter- and intrafamilial variability in the other manifestations of PT dysfunction, which may cause a renal FS with hypophosphatemic rickets, as well as in the extent of nephrocalcinosis/nephrolithiasis.

In addition to LMW proteinuria (i.e., urinary excretion of proteins, such as α1 and β2 microglobulins, retinol-binding protein, Clara cell protein, and vitamin D–binding protein), the manifestations of PT dysfunction in Dent disease may include aminoaciduria, phosphaturia, glycosuria, uricosuria, kaliuresis, and impaired urinary acidification, being often complicated by rickets or osteomalacia.[64] These features are generally found in males only, who may have manifestations of the disease from early childhood.[67,68,71] Median age at diagnosis was 9 (0.2–67) years in 377 male patients with mutation-proven disease.[70] These patients may present with bone pain and difficulty in walking due to rickets, or symptoms of renal stones, such as abdominal pain and hematuria. Occasionally, patients are referred following the fortuitous discovery of biological manifestations of PT dysfunction. Hypercalciuria and nephrocalcinosis occur in 95% and 75% of affected males respectively. There is considerable variability in the occurrence of nephrolithiasis; it occurs in ~50% of affected males. Progression to end-stage renal failure occurs between the 3rd and the 5th decades of life in 30%–80% of affected males.[67] Additional clinical and biological abnormalities described in patients with Dent disease[70] include micro- or macrohematuria, urinary concentration defects with polyuria/nocturia, proteinuria in the nephrotic range[72] and biopsy-proven focal glomerulosclerosis,[73] hypomagnesemia, secondary aldosteronism with Bartter-like phenotype,[74–76] and night blindness responsive to vitamin A.[77]

Female carriers present with the clinical manifestations of Dent disease, reflecting the degree of X chromosome inactivation. In particular, LMW proteinuria (60%), hypercalciuria (31%), nephrolithiasis (21%), and nephrocalcinosis (12%) were reported in a cohort of 95 heterozygous women.[70] The extent of LMW proteinuria is approximately 50× lower than in affected males. Thus far, end-stage renal disease has been reported in only 1 female carrier,[64] whereas CKD stage 3 has been reported in three additional heterozygous women.[70]

7.1.2 Dent Disease 2

A small subset of patients with Dent disease present extrarenal manifestations, such as developmental delay, hypotonia, and cataract, and such patients have been reported to share mutations in *OCRL* with the oculocerebrorenal syndrome of Lowe.[69,78] The occurrence of these extrarenal manifestations with mutations associated with Lowe syndrome is referred to as Dent disease 2. Patients with Dent disease 2 show hypercalciuria and LMW proteinuria; they may also present with nephrocalcinosis, nephrolithiasis, hematuria, hypophosphatemia, and/or renal insufficiency. Only a minority of these patients have been observed to have mild intellectual deficit, hypotonia and subclinical cataract. Mild phenotype actually dissuaded the clinicians from considering a diagnosis of Lowe syndrome, usually associated with congenital cataracts, delayed motor milestones, intellectual impairment in almost all affected males, growth retardation, rickets, and renal proximal tubulopathy. Moreover, the patients with Dent disease 2 and mild intellectual deficit were adults, who had not developed overt features of Lowe syndrome.[69,78]

7.2 Genetics

Dent disease may be caused by either inactivating mutations in *CLCN5* (OMIM *300008), which maps on chromosome Xp11.22 and encodes a 746 amino-acid electrogenic Cl^-/H^+ exchanger (ClC-5),[65,79] or the *OCRL* gene (OMIM *300535), which maps on chromosome Xq25 and encodes the phosphatidylinositol 4,5-biphosphate 5-phosphatase OCRL.[78]

Approximately 50%–60% of patients with Dent disease harbor mutations in the *CLCN5* gene (Dent disease 1). A total of 234 mutations in the *CLCN5* gene have been reported in 413 families.[68,70] Family studies revealed a de novo mutation rate of approximately 12%.[70] Dent disease is reported all over the world, with higher rates of detection in Japan reflecting a systematic program of LMW protein screening in children.[66] The mutations of *CLCN5* are scattered throughout the coding region. There is no correlation between the mutations and phenotypes and/or between the presence or absence of a *CLCN5* mutation and the Dent's disease phenotype. The 234 *CLCN5* mutations include large deletions (4.7%), nonsense, and frameshift mutations that produce premature stop codons (18% and 29%), splice-site mutations predicted to interfere with correct splicing (12%), missense and small in-frame deletions affecting conserved AA residues (33% and 2.6%, respectively).[70] The majority of mutations are predicted to result in truncated or absent ClC-5 protein, which would lead to complete loss of antiporter function. Heterologous expression of the *CLCN5* mutants has revealed that the majority of these alleles lead to a loss of Cl^- conductance.[65] Type-I mutants underwent complex glycosylation and traffic normally to the cell surface and to early endosomes, but exhibit significant reductions in ion currents, whereas type-II mutants showed improper glycosylation and were nonfunctional due to retention in the endoplasmic reticulum.[80,81] Clustering of the missense mutations at the interface between the two subunits has been reported, emphasizing the functional importance of ClC-5 homodimerization.[82]

Approximately 15% of patients with Dent disease have mutations in the *OCRL* gene.[69,70,78,83] These patients (Dent disease 2) are clinically indistinguishable from those that have *CLCN5* mutations.[69] None of these patients had the severe cataracts or intellectual deficit that is typically found in patients with Lowe syndrome. Furthermore, the *OCRL* mutations associated

with Dent disease 2 do not overlap with those causing Lowe syndrome. All of the *OCRL* missense mutations associated with Dent disease occur in the 5′ region of the gene (exons 4–15) and involve the phosphatidylinositol phosphate 5-phosphatase domain of the OCRL protein, while the truncating mutations are in the first 7 exons or intron 7. By contrast, the *OCRL* mutations that are found in Lowe syndrome patients occur primarily in exons 9–22, which encode the 3 large functional domains.[69] Coinheritance of a truncating mutation in the *CLCN5* gene and a donor splice-site alteration in the *OCRL* gene has been reported in a patient with a phenotype (dysmorphic features, peripapillary optic nerve atrophy, aminoaciduria, hypophosphoremia, hypercalciuria, LMW proteinuria, growth delay, florid rickets, mild mental retardation, and attention deficit hyperactivity) close to Lowe syndrome.[84]

Till present, 25%–35% patients with Dent disease have neither *CLCN5* nor *OCRL* mutations but possibly defects in other genes that could code for functionally related proteins. No mutations in *CLCN4* (ClC-4), *CFL1* (cofilin-1), *SLC9A6* (NHE6), and *TMEM27* (collectrin) have been identified.[68,78,85,86]

7.3 Pathophysiology

7.3.1 Dent Disease 1

The complex phenotype of Dent disease 1 is probably explained by the predominant expression of ClC-5 in PT segments, with more discrete expression in the thick ascending limb of Henle's loop and the α-type intercalated cells of the collecting ducts of the kidney.[87] In PT cells, ClC-5 codistributes with the vacuolar H^+-ATPase (V-ATPase) in early endosomes,[87,88] which are responsible for the reabsorption and processing of albumin and LMW proteins that are filtered by the glomerulus. These vesicles belong to the receptor-mediated endocytic pathway, which involves the multiligand receptors, megalin, and cubilin, located at the apical brush border of PT cells.[7,89] Progression along the endocytic apparatus depends on endosomal acidification, driven by the V-ATPase and requiring a parallel Cl^- conductance to neutralize the vesicular membrane potential (Fig. 41.1). Accordingly, the loss of the endosomal Cl^- conductance would impair vesicular acidification, causing dysfunction of PT cells.[89] Two independent strains of ClC-5 KO mice have been generated, which both recapitulate the major features of Dent's disease, including LMW proteinuria and other manifestations of the renal FS.[90,91] Furthermore, in vitro experiments have shown a decreased acidification of early endosomes in ClC-5 KO mice.[92]

However, ClC-5 is a $2Cl^-/H^+$ exchanger rather than a Cl^- channel.[79] Jentsch et al. compared a knock-in (KI) mouse harboring a point mutation in a critical glutamate residue, which converts the exchanger into an uncoupled Cl^- channel (that should facilitate endosomal acidification) with a conventional ClC-5 KO mouse.[92] As expected, the acidification of renal endosomes from wild-type and KI mice was normal, but severely impaired in KO mice. Surprisingly, despite normal endosomal acidification, KI mice showed the same renal phenotype as KO mice and patients with Dent disease, including LMW proteinuria, glycosuria, hyperphosphaturia, and hypercalciuria. Furthermore, both the KI and KO mouse showed impaired PT endocytosis, indicating that PT dysfunction in Dent's disease may occur despite normal endosomal acidification. Studies in conditionally immortalized tubular cells from patients harboring *CLCN5* mutations also showed different effects on endosomal acidification, uncoupled to defects in receptor-mediated endocytosis.[93] Taken together, these findings may suggest that impaired regulation of endosomal Cl^- concentration could play a role in the PT defects associated with Dent disease.[92]

Studies in mice have demonstrated that inactivation of ClC-5 is associated with a severe trafficking defect in PT cells, with loss of megalin and cubilin at the brush border, subsequent loss of their ligands in the urine, and impaired lysosomal processing.[90,91,94] As the megalin/cubilin complex mediates the reabsorption of the ultrafiltrated vitamin D–binding protein, 25(OH)-vitamin D3 and PTH, the urinary loss of these mediators could potentially lead to variable levels of active $1,25(OH)_2$-vitamin D_3 levels in the serum. In turn, such variability could explain the variable hypercalciuria and kidney stone formation observed in mouse models[90,91] and patients harboring ClC-5 mutations.[67] The deletion of ClC-5 in mouse and human PT cells is associated with increased cell proliferation, oxidative stress, and the specific induction of type III carbonic anhydrase.[95] Furthermore, ClC-5 inactivation is associated with impaired lysosome biogenesis, which contributes to defective endocytosis and urinary loss of lysosomal enzymes.[96]

Potential roles of ClC-5 in the more distal nephron segments remain to be defined. The hypercalciuria observed in patients with Dent's disease and some ClC-5-deficient mice may be secondary to the PT dysfunction (urinary loss of vitamin D–binding protein and reduced phosphate absorption, leading to increased $1,25(OH)_2$-vitamin D_3 synthesis) or, at least in part, caused by the functional loss of ClC-5 in the thick ascending limb. A small fraction of patients with Dent's disease may have nephrocalcinosis without hypercalciuria,[71] which could indeed reflect the fact that ClC-5 is distributed in several nephron segments that can contribute to the genesis of kidney stones through different mechanisms. For instance, it has been suggested that collecting duct cells lacking ClC-5 may show an impaired ability of internalization of calcium crystals adhering to apical cell surface.[97]

7.3.2 *Dent Disease 2*

The phenotype of Dent disease 2 due to *OCRL* mutations may in part be attributed to the role of *OCRL* in lysosomal trafficking and endosomal sorting. *OCRL* encodes a member of the type II family of inositol polyphosphate 5-phosphatases.[98] These enzymes hydrolyze the 5-phosphate of inositol 1, 4,5-trisphosphate, and of inositol 1,3,4,5-tetrakisphosphate, PIP_2, and phosphatidylinositol 3,4,5-trisphosphate, thereby presumably inactivating them as second messengers in the phosphatidylinositol signaling pathway. The preferred substrate of OCRL is PIP_2, and this lipid accumulates in the renal PT cells of patients with Lowe syndrome.[99] OCRL is localized to lysosomes in renal PT cells and to the trans-Golgi network in fibroblasts. This localization is consistent with the role of OCRL in lysosomal enzyme trafficking from the trans-Golgi network to lysosomes, and the activities of several lysosomal hydrolases are found to be elevated in the plasma of affected patients.[100] OCRL has also been shown to interact with clathrin and indeed colocalizes with clathrin on endosomal membranes that contain transferrin and mannose-6-phosphate receptors.[28] Mannose-6-phosphate receptor-bound lysosomal enzymes are recruited by appendage subunits and Golgi-localized binding proteins into clathrin-coated vesicles that transport them from the trans-Golgi network to endosomes. OCRL also interacts with the Rab5 effector APPL1, thus potentially playing a role in the early endocytic pathway.[101] Thus, it seems likely that the *OCRL* mutations in Lowe syndrome patients result in OCRL protein deficiency, which leads to disruptions in the endosomal and/or lysosomal trafficking. This abnormality is similar to that observed in Dent disease 1, and it seems that Dent disease therefore may be due to abnormalities in either endosomal acidification and sorting, or lysosomal trafficking. It must be noted that the targeted disruption of the murine ortholog for OCRL does not cause Lowe syndrome, because *Ocrl* deficiency is complemented in mice by inositol polyphosphate 5-phosphatase (*Inpp5b*).[102] This difficulty was contoured by replacing mouse *Inpp5b* with human *INPP5B* in *Ocrl*-deficient mice, which provided the first mouse model recapitulating Lowe syndrome and Dent disease 2 caused by the deficiency in OCRL.[103]

7.4 Diagnostic Aspects

The clinical diagnosis of Dent disease is based on the presence of all three of the following criteria:

1. LMW proteinuria (elevation of urinary excretion of β2-microglobulin, Clara cell protein, and/or RBP by at least fivefold above the upper limit of normality);
2. hypercalciuria (>4 mg/kg in a 24 h collection or >0.25 mg Ca^{2+} per mg creatinine on a spot sample); and
3. at least one of the following: nephrocalcinosis, kidney stones, hematuria, hypophosphatemia, or renal insufficiency.

The clinical diagnosis is supported by a history of X-linked inheritance of renal FS and/or nephrolithiasis. The identification of mutation in either *CLCN5* or *OCRL* confirms the diagnosis. However, some patients with *CLCN5* mutations have been reported to have LMW proteinuria or hypercalciuria alone,[104,105] and thus in the presence of an identified *CLCN5* mutation, only one of the above clinical criteria may be sufficient to establish an affected status in an individual. It is important to note that the absence of clinical cataracts and the lack of severe intellectual deficit are key features that make a diagnosis of Dent disease 2, associated with *OCRL1* mutations, more likely than a diagnosis of Lowe syndrome.

There have been few reports of renal biopsies in cases with proven *CLCN5* mutations.[106] Light microscopy studies revealed progressive, non-specific lesions that include glomerular hyalinosis, tubular cell degeneration or atrophy, and mild interstitial fibrosis. Of interest, these kidneys invariably showed hyaline casts that were sometimes calcified, located in the outer medulla and presumably the first manifestations of nephrocalcinosis. By contrast, electron microscopy did not reveal any ultrastructural abnormalities in PT cells.

The differential diagnosis of Dent disease includes the other causes of renal FS, that can be inherited, acquired or caused by exogenous substances (Table 41.1).

7.5 Genetic Counseling

Both forms of Dent disease are X-linked: males are thus affected more severely and females, who are only mildly affected, are carriers and will transmit the disease to half of their sons whereas half of their daughters will be carriers. All the daughters of affected males will be carriers, but all the sons of affected fathers will be normal. In approximately 10% of patients, Dent disease occurs de novo, but the disease will be transmitted as an X-linked trait to subsequent generations.[70] If the mother or siblings of a patient with Dent's disease are eager to know their genetic status, then mutational analysis of *CLCN5* and/or *OCRL* using leukocyte DNA can be undertaken. Although technically feasible, antenatal diagnosis and preimplantation genetic testing for Dent's disease is not advised, and as yet has not been requested, because the vital prognosis in the majority of patients is good and there is no evidence for a genotype–phenotype correlation. Indeed the severity of the disease may vary considerably in individuals from the same family.[70,71]

7.6 Treatment

In the absence of therapy targeting the molecular defect, the current care of patients with Dent disease is supportive, focusing on the prevention of nephrolithiasis and metabolic manifestations. Thiazide diuretics can be used to treat hypercalciuria,[107,108] although significant adverse events, including hypovolemia and hypokalemia related to the primary tubulopathy, have been reported.[108] Similarly, treatment of rickets with vitamin D must be cautious since it may increase hypercalciuria. Studies performed on ClC-5-deficient mice suggest that long-term control of hypercalciuria by a high citrate diet delays progression of renal disease even in the apparent absence of stone formation.[109]

8 BARTTER'S SYNDROME

In 1962, Bartter and co-workers described two African American patients with a syndrome characterized by hypokalemic metabolic alkalosis, renal K^+ wasting, hypertrophy of the juxtaglomerular apparatus, normotensive hyperaldosteronism, increased urinary excretion of prostaglandins, and high plasma renin activity.[110] Several phenotypic variants have been progressively identified, to constitute a group of hypokalemic salt-losing tubulopathies, refered to as Bartter-like syndromes.[111] All these disorders are recessively inherited and associated with hypokalemia and hypochloremic metabolic alkalosis due to stimulation of the renin–angiotensin–aldosterone system. However, they markedly differ in terms of age of onset, severity of symptoms, presence of urinary concentrating defect, other electrolyte abnormalities (e.g., hypomagnesemia), and magnitude of urinary calcium excretion.

Based on clinical manifestations, the Bartter-like syndromes were grouped into two major groups: the antenatal Bartter syndrome (aBS) [also named hyperprostaglandin-E syndrome (HPS)], which can be associated or not with sensorineural deafness (SND), and the classic Bartter syndrome (cBS).[112] The aBS is caused by inactivating mutations in the *SLC12A1* gene encoding the apical sodium–potassium–chloride cotransporter NKCC2[113] or the *KCNJ1* gene coding for the potassium channel ROMK.[114] Inactivating mutations in *BSND* which codes for barttin, a regulatory β-subunit of the basolateral ClC-Ka and ClC-Kb channels, were detected in aBS with SND.[115] Inactivating mutations of *CLCNKB* which encodes the chloride channel ClC-Kb are associated with cBS.[116] A classification of these salt-losing tubulopathies, based on clinical, physiological, and genetic informations is shown on Table 41.3.

Typical features of aBS type I (NKCC2) and type II (ROMK) include marked polyhydramnios, premature delivery, massive polyuria, life-threatening episodes of dehydration, and hypercalciuria leading to nephrocalcinosis within the first months of life. Failure to thrive and growth retardation are invariably observed. Systemic manifestations, including fever of unknown origin, diarrhea, vomiting, generalized convulsions, have been attributed to overproduction of PGE.[117] Osteopenia is common in aBS,[118] associated with high urinary excretion of bone resorption markers.[119] Hypophosphatemia with decreased tubular phosphate reabsorption has been described, possibly related to tubular damage and hypokalemic nephropathy.[119]

Patients harboring mutations in barttin show the most severe form of aBS. All patients are deaf and show a severe growth defect, with delayed motor development.[120] Patients harboring mutations of ClC-Kb present a broad spectrum of manifestations ranging from the aBS/HPS phenotype with polyhydramnios, isosthenuria, and hypercalciuria over the classic BS phenotype with less impaired concentrating ability and normal urinary calcium excretion to a Gitelman syndrome-like phenotype with hypocalciuria and hypomagnesemia.[111,121] Two cases of severe BS with sensorineural deafness in children caused by simultaneous mutations in both the

TABLE 41.3 Inherited Bartter-Like Salt-Losing Tubulopathies

Disorders	OMIM #	Loci	Genes	Proteins
aBS, HPS, Type I BS[a]	601678	15q15-q21.1	*SLC12A1*	Na^+–K^+–$2Cl^-$ cotransporter NKCC2
aBS, HPS, Type II BS[a]	241200	11q24	*KCNJ1*	K^+ channel ROMK (Kir1.1)
aBS with SND[b], type IVA BS[a]	602522	1p31	*BSND*	Barttin, β-subunit of ClC-Ka/b
cBS type III BS[a]	607364	1p36	*CLCNKB*	Cl^- channel ClC-Kb

aBS, Antenatal Bartter syndrome; BS, Bartter syndrome; cBS, classic Bartter syndrome; HPS, hyperprostaglandin-E syndrome; OMIM, online Mendelian inheritance in man; SND, sensorineural deafness.

[a]*This classification is based on the chronological order of gene discovery.*

[b]*A digenic disorder with inactivating mutations of CLCNKA and CLCNKB has been associated with the aBS with SND phenotype. This digenic disorder has been named BS type IVB, OMIM #613090.*

CLCNKA and *CLCNKB* genes (BS type 4B) have also been described.[122,123]

Differential diagnosis, rare associations, and treatment of BS has been reviewed in detail.[117] As mentioned earlier, a few patients harboring *CLCN5* mutations may present with a Bartter-like syndrome.[75–77] The appropriate management of aBS with correction of fluid and electrolyte disorders, indomethacin, and K^+ supplements results in catch-up growth and normal pubertal and intellectual development.[124] However, most patients show a persistent deficit in height and weight.[125]

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CHAPTER

42

Inherited Magnesium Disorders

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1 INTRODUCTION

The physiology of magnesium homeostasis has been studied for decades. In recent years, however, discoveries made through studies of genetic disorders have provided remarkable insights into the molecular determinants of this process.

Magnesium is essential to a wide range of physiologic functions. After potassium it is the next most abundant intracellular cation. It is a major determinant of neuromuscular excitability. It plays a role in the regulation of ion channel activities. It is essential to proper bone mineralization. Magnesium is a critical cofactor in enzymatic reactions, such as those for synthesis of DNA and proteins and in energy metabolism. Magnesium adequacy is necessary for normal placental development and fetal growth.[1] Thus, its levels are regulated within a narrow range. The body's magnesium balance is set by the processes of intestinal absorption, bone mineralization, and renal excretion. Of these, renal magnesium reabsorption is particularly tightly regulated to maintain this balance.

Roughly one-third of ingested magnesium is absorbed, principally in the small bowel. Absorption is partially offset by intestinal magnesium secretion, so that net absorption amounts to approximately 100 mg (4.1 mmol) per day, which equals urinary excretion when in balance.[2]

Intestinal magnesium reabsorption involves two transport systems: active transcellular transport that is critical at low concentrations of magnesium but is saturable at higher concentrations in the lumen of the gut, and passive paracellular transport at high luminal magnesium concentrations.[3,4] The first step in the transcellular transport process involves magnesium entry into the cell mediated by a channel encoded by the *TRPM6* gene, which serves the same function in the distal convoluted tubule (DCT).[5] Dysfunction of this channel is associated with both intestinal malabsorption of magnesium and failure of renal magnesium conservation in the hereditary syndrome of hypomagnesemia with secondary hypocalcemia (HSH).

Changes in magnesium absorption are matched by changes in urinary magnesium reabsorption, which occur primarily in the ascending limb of Henle's loop and in the distal tubule. In contrast to the handling of sodium or calcium, only about 15% of filtered magnesium is reabsorbed in the proximal renal tubule. Sixty to seventy percent is reabsorbed in the thick ascending limb (TAL) of Henle's loop and 10% in the DCT. Between 3% and 5% is excreted in the urine, though in states of magnesium deprivation urinary excretion can be lowered to less than 1% of the filtered magnesium.[5] Magnesium reabsorption in the TAL is a passive paracellular process driven by the lumen-positive electrical gradient. Proteins involved in this process are illustrated on Fig. 42.1. The tight junction proteins claudin-16 and -19 are important in this paracellular pathway, and are mutated in familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC).[6] The gradient providing the driving force is produced by the activities of several transport proteins. Active sodium reabsorption results from activities of the apical Na^+-K^+-$2Cl^-$ cotransporter (NKCC2) and the basolateral Na^+/K^+-ATPase. These are both processes in which transported charges are balanced (electroneutral). The apical potassium channel ROMK allows recycling of potassium back into the lumen down its chemical gradient, and the basolateral chloride channel ClC-Kb mediates efflux of chloride, both electrogenic processes contributing to the net potential difference across the epithelial layer that drives passive cation reabsorption. Activation of the Ca^{++}-sensing receptor (CaSR) inhibits this process and reduces the driving force favoring paracellular reabsorption of both calcium and magnesium[5,7] Several of these proteins are mutated in the Bartter syndrome; of these, mutations in the genes encoding ClC-Kb and the CaSR are more likely to be associated with hypomagnesemia.[8]

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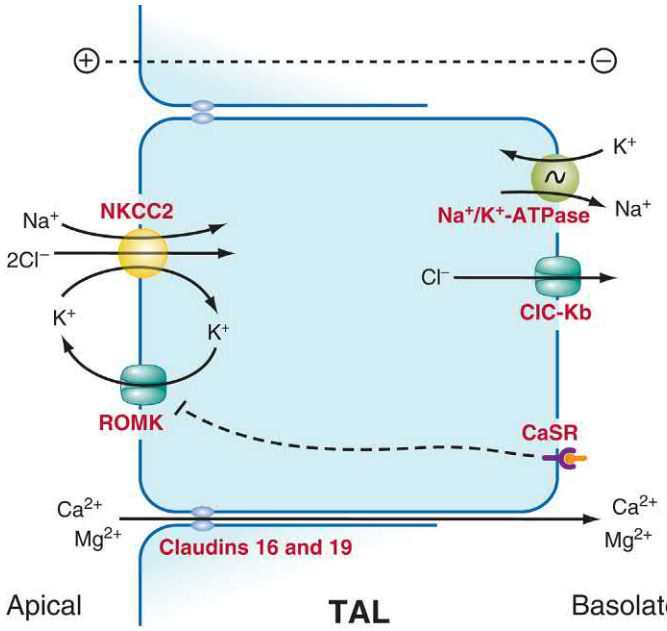

FIGURE 42.1 Schematic representation of cells of the thick ascending limb (TAL) of Henle's loop, depicting proteins related to magnesium transport. See text for explanation.

Final modulation of magnesium reabsorption in the DCT establishes the level of magnesium excretion in the urine. The proteins involved in these processes are depicted on Fig. 42.2. In the DCT, magnesium reabsorption is an active transcellular process. Lumenal magnesium enters the cell across the apical magnesium channel TRPM6, the gene for which is mutated in HSH. The driving force for magnesium entry across this channel, without a chemical gradient, is the potential difference across the apical membrane (lumen-to-intracellular), which is dependent on the potassium channel Kv1.1. This channel is encoded by the gene *KCNA1*, which is mutated in dominant hypomagnesemia with episodic ataxia.[9] Activation of the receptor for the epidermal growth factor (EGF) stimulates trafficking of TRPM6 to the target apical membrane and increases the activity of the transporter.[7] EGF is mutated in isolated autosomal recessive hypomagnesemia (IRH).[10]

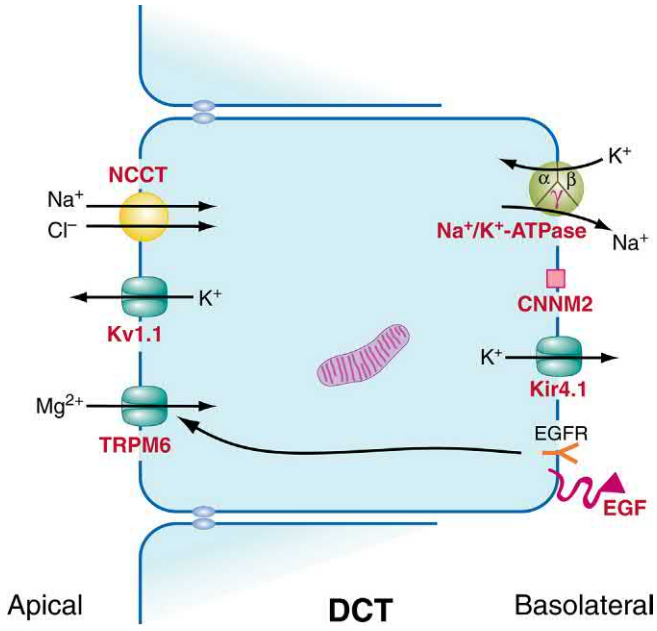

FIGURE 42.2 Schematic representation of cells of the distal convoluted tubule (DCT), depicting proteins related to magnesium transport. See text for explanation.

Basolateral magnesium efflux is sodium-dependent, but the transporter is not known. CNNM2 is a protein expressed on the basolateral surface of DCT cells that is mutated in rare patients with inherited hypomagnesemia, but whether it functions as a transport protein, magnesium sensor, or for some other function, is unknown;[8] it appears not to be a magnesium transporter.[11]

Sodium reabsorption in the DCT involves apical cotransport of sodium and chloride by the sodium-chloride cotransporter NCCT and extrusion of sodium in exchange for potassium by the basolateral Na^+/K^+-ATPase. The Kir4.1 basolateral potassium channel may serve to assure an adequate potassium supply for the Na^+/K^+-ATPase. Mutations in *NCCT* or in the gene (*FXYD2*) encoding the γ subunit of the Na^+/K^+-ATPase are associated with Gitelman syndrome (GS)[12] and autosomal dominant hypomagnesemia,[13] respectively. Mutations in *KCNJ10*, encoding Kir4.1, have been reported in a syndrome of hypomagnesemia associated with deafness and neurologic abnormalities.[14,15]

2 FAMILIAL HYPOMAGNESEMIA WITH HYPERCALCIURIA AND NEPHROCALCINOSIS (FHHNC)

2.1 Clinical Features

FHHNC is a rare autosomal recessive disorder caused by mutations in two genes, *CLDN16* and *CLDN19* that encode two of the tight junction proteins of the claudin multigene family, claudins-16 and -19. The clinical disorder has been recognized since the first report by Michelis et al. in 1972 of a family affected with hypomagnesemia and excessive renal wasting of magnesium and calcium (Michelis–Castrillo syndrome).[16]

Patients typically present symptomatically in childhood with recurrent urinary tract infections, polyuria and/or polydipsia.[17] Other symptoms include kidney stones, vomiting, abdominal pain, failure to thrive, tetany, convulsions, gouty arthritis, and defective hearing. Urinary magnesium wasting is severe, and serum magnesium levels are depressed to the range of 1 mEq/L.

Renal insufficiency, often present even at the time of diagnosis, distinguishes FHHNC from other inherited hypomagnesemic syndromes. One-third of patients have chronic kidney disease (CKD; GFR <60 mL/min/1.73 m^2) by adolescence.[17] The rate of decline of renal function correlates with the degree of nephrocalcinosis.[18] However, Konrad and coworkers point out that in other inherited tubulopathies, such as antenatal Bartter syndrome,[19] nephrocalcinosis even when severe does not always correlate with renal failure. This is also the case in Dent disease.[20] Recurrent urinary tract infection, a common problem in FHHNC, may well contribute to renal insufficiency.[19] The recent observation of a correlation between the aggressiveness of renal decline and the nature of mutation in the disease gene, discussed later, supports the suggestion that the gene defect may contribute directly to renal failure.[21]

Serum parathyroid hormone levels are elevated in most patients even without renal failure, reflecting both the excessive loss of calcium and the stimulatory effect of hypomagnesemia on PTH secretion.[17,18] Other metabolic abnormalities include hypocitraturia, impaired urinary acidification, and hyperuricemia.[19]

Visual impairment is the most common extrarenal abnormality in FHHNC. Eye findings are mild or absent in those with mutations in *CLDN16*, but patients with mutations in *CLDN19* develop significant abnormalites such as macular coloboma (misdevelopment of the optic disc) that can cause severe visual impairment; other abnormalities include horizontal nystagmus and myopia.[18,22,23] Another extrarenal consequence, seen in patients with mutations in either gene, is bone demineralization even overt rickets, a consequence of urinary calcium loss. Hearing impairment and dental anomalies are also found in a subset of patients.

Defective tooth enamel (amelogenesis imperfect) has been described in patients with mutations in *CLDN16*[24] and *CLDN19*.[25]

2.2 Molecular Genetics of Claudins-16 and -19

The gene initially named Paracellin-1 (*CLDN16*, encoding claudin-16) was identified by Lifton's lab in 1999 by genetic mapping in 12 kindreds with FHHNC (10 of them consanguineous) to a region on chromosome 3 (3q27-19), and identification of the gene by positional cloning.[6] Since then, over three dozen distinct *CLDN16* mutations have been identified in over 70 patients with FHHNC. Studies by Weber and coworkers in subjects with the renal phenotype of FHHNC but lacking mutation in *CLDN16* identified a region on chromosome 1p34.2 that harbored *CLDN19*, which was mutated in these patients. The latter group had severe eye abnormalities, as described previously.[26]

Consistent with the observed autosomal recessive inheritance, clinically affected individuals are either homozygous for a particular mutation, or compound heterozygotes with two different allelic mutations in the same gene. Although carriers are typically asymptomatic, an observed increased incidence of hypercalciuria, stones[17,18] and mild hypomagnesemia[21] in first-degree relatives of affected probands suggests a possible phenotype for some carriers.

The number of known mutations in *CLDN16* is now sufficient to have allowed for the discovery of a correlation between the nature of mutation and the severity of phenotype in FHHNC. Konrad et al. separated mutations that cause a complete loss of claudin function as manifested either in expression studies or as nonsense, truncating, and splice-site mutations, from those that leave substantial residual function (>40% function). They found that patients with two complete loss-of-function mutations had an earlier age of onset and more rapid decline of renal function than those with either two partial loss-of-function mutations or with one partial and one complete loss-of-function mutation.[21] In contrast, one patient with a homozygous multiexon deletion in *CLDN16* (exons 2–5), presumed (though not determined) to alter function substantially if not completely, remained healthy with normal growth through age 20, though ultimately progressing to end-stage renal failure.[27]

2.3 Claudins and Renal Physiology

The term claudin was taken from the latin claudere, "to close" based on observations in the first detected claudins, claudins-1 and -2, which exhibited a sealing function.[28] Many of the 24 known claudins allow

selective solute movement across the sealed barrier, including claudins-16 and -19. In the kidney, both claudins-16 and -19 are expressed predominantly in the thick ascending loop of Henle (TAL), the major site of passive paracellular reabsorption of divalent cations, but also in the DCT.[19] Consistent with the clinical phenotypes, claudin-19 is also expressed in the eye, but claudin-16 is not.

Epithelial tight junctions serve as a diffusion barrier to fluid and solutes but also allow selective permeability to specific ions. They are formed as complexes of proteins, such as occludin and tricellulin, as well as claudins. Claudin structures all contain 4 transmembrane helices, 2 extracelluar loops, and intracellular amino and carboxy termini. The first loop contains many negatively charged residues and is thought to determine paracellular charge selectivity; most missense mutations in *CLDN16* and all reported mutations in *CLDN19* are in this first extracellular loop. The second loop appears important in forming tight junction strands through interaction with other claudins.[19,26]

In the tight junction the interaction between claudins-16 and -19 establishes cation selectivity.[29] However, whether these claudins themselves represent the selective pore for calcium and magnesium is not clear; for example, when expressed in LLC-PK1 cells claudin-16 has a much greater effect on permeability for sodium than for magnesium.[30] Using a novel approach with high-resolution scanning ion conductance microscopy, Gong and coworkers characterized the claudin 16/19 dimer and concluded that it does not constitute a channel.[31] Recently, Gunzel et al. have proposed that claudin-16 enhances magnesium transport by altering the potential difference, that is, the driving force for reabsorption, through changes in chloride currents.[32] However, since the cell model they transfected, MDCK cells, are a high-resistance cell line more resembling DCT than TAL cells, it is not clear that these observations are relevant to the site of the defect in FHHNC, which is TAL.[32]

Recent studies have elucidated a role for claudin-14 in mediating the regulation by extracellular calcium of cation transport in the TAL. Activation of the CaSR suppresses the cation selectivity of the claudin- 16/19 complex through modulation of claudin-14.[33] In mice, knockout of *CLDN14* produces a phenotype of hypocalciuric, hypomagnesiuric hypermagnesemia, the mirror image of the human FHHNC,[33] and overexpression of *CLDN14* in mice produces a phenotype duplicating human FHHNC.[34] Such findings, and others, are consistent with a Scandinavian genome-wide association study implicating population variants in human *CLDN14* in human hypercalciuric stone disease and reduced bone mineral density[35] and another population study in which *CLDN14* variants were associated with calcium excretion.[36] Mutations in *CLDN14* have been reported to cause autosomal recessive deafness, but to date have not been reported to be associated with hereditary magnesium disorders.[37]

2.4 Animal Models

Consequences of loss of function of these claudins have been studied in mouse models and have confirmed the role of these genes in the disease phenotype. Knockdown mice produced using small interfering RNA (siRNA) with reduced expression of either claudin-16 or claudin-19 demonstrate the absence of the other claudin at the tight junction.[38] This confirms the importance of the interaction between these two claudins in forming appropriate junctions. Both knockdown models manifest hypomagnesemia, with urinary wasting of magnesium and calcium.[38,39] Claudin-16 knockdown mice also exhibit medullary calcification, as in humans.[39]

Renal function has also been studied in a model in which the *CLDN16* gene is inactivated by knockout. Absence of claudin-16 in this knockout model also results in hypomagnesemia and hypercalciuria, with compensatory increases in parathyroid hormone and 1,25-dihydroxyvitamin D, as is found in patients with FHHNC. As in the knockdown models, *CLDN16* knockout mice have significantly reduced expression of claudin-19.[40]

In Japanese Black (Wagyu) cattle a disease characterized by renal failure with chronic interstitial nephritis and diffuse fibrosis is inherited in an autosomal recessive fashion. Breeding and mapping studies identified the region responsible as harboring the bovine gene for claudin-16 with deletion of the first four exons.[41,42] These cows develop renal tubular dysplasia at an early age, suggesting that claudin-16 may play a role in normal nephron development. There was a tendency toward lower serum calcium levels in the affected cattle but not significance, and no apparent difference in serum or urine magnesium levels, though it is not clear whether these measurements were made before the onset of renal failure.[42] Magnesium physiology is different in ruminants, in whom the gastrointestinal tract plays a significant role in magnesium excretion.[42]

2.5 Diagnosis and Treatment

The combination of hypomagnesemia with hypercalciuria and nephrocalcinosis is distinctive, and particularly when in combination with eye abnormalities. Clinically significant hypomagnesemia is not characteristic of Dent disease or of distal renal tubular acidosis, two other inherited conditions with nephrocalcinosis. Further, if sufficient information on family members is available, evidence of autosomal inheritance would exclude Dent disease, which is X-linked. Mutation analysis of *CLDN16* and *CLDN19* is not currently available for clinical diagnosis.

Treatment of FHHNC is currently aimed at addressing symptoms. Intervention to treat urinary infections and prevent recurrences is important. Correction of hypomagnesaemia with magnesium supplementation should be attempted but is often unsuccessful because of the magnitude of urinary losses. A metabolic evaluation of stone risk is warranted, and those factors identified, particularly hypercalciuria, should be addressed. A short-term study demonstrated a reduction in urinary calcium excretion in FHHNC with hydrochlorothiazide.[43] However, the benefit of thiazides on stone recurrence or progression of renal failure in patients with FHHNC is not known. Sodium balance is maintained in FHHNC, so sodium intake should be restricted in an attempt to enhance distal mineral reabsorption.

In the long run, renal transplantation is justified in patients with renal failure. Nephrocalcinosis has not been reported to recur after kidney transplant in FHHNC, and calcium and magnesium losses do not recur, consistent with a primary renal defect rather than a systemic process.[18,19]

3 PRIMARY HYPOMAGNESEMIA AND SECONDARY HYPOCALCEMIA (HSH)

HSH (MIM 602014) is an autosomal recessive disorder of intestinal and renal magnesium transport caused by mutations in the *TRPM6* gene encoding an ion channel involved in renal and intestinal cation transport. The clinical syndrome was initially described by Paunier et al. in 1968.[44] Patients typically present within the first 3 months of life with increased neuromuscular activity: seizures, tetany, and muscle spasms, neurologic consequences of hypomagnesemic hypocalcemia; it has also been referred to as hypomagnesemic tetany. Hyperuricemia and hypocitraturia are also features.[45] Without treatment, the condition may cause permanent neurologic damage or even death.

The primary defect is impaired intestinal magnesium absorption.[3,44] Serum levels of magnesium are severely depressed, typically to below 0.35 mM, and calcium to below 2.0 mM. Fractional magnesium excretion is high, evidence of a renal magnesium leak.[46,47] The hypocalcemia is in striking contrast to the normal serum calcium levels in FHHNC. Despite hypocalcemia, circulating PTH levels are low or even undetectable, reflecting failure of parathyroid hormone release as a consequence of sustained magnesium deficiency.[48] While moderate degrees of hypomagnesemia stimulate PTH release, severe magnesium depletion, as occurs in HSH, as well as in chronic alcoholism with malnutrition, impairs both secretion of PTH and its effect on target tissues by interfering with signal transduction.[49,50] This functional hypoparathyroidism exacerbates the hypocalcemia. Magnesium replacement immediately restores serum levels of both calcium and PTH.

It was initially proposed that HSH is X-linked, based on a preponderance of affected males in early reports.[51] This seemed to be confirmed by the observation of a balanced X:9 translocation.[52] However, Walder et al., mapped the disease locus in three inbred Bedouin kindreds from Israel, localized the disease gene to 9q22 (where these kindreds were homozygous for a shared disease haplotype, establishing a common ancestry). Applying their genetic markers to DNA amplified from a cell line containing the previously-reported X:9 translocation confirmed that the flanking markers encompassed the breakpoint.[53] Their group and another reported simultaneously in 2002 mutations in the *TRPM6* gene in this region that encodes an ion channel belonging to a subgroup of the transient receptor potential (TRP) channel family;[54,55] the range of mutations has been expanded by others as well, the majority of which are truncating mutations.[56–62] Thus, HSH is an autosomal recessive disease inherited on chromosome 9.

3.1 Physiology of TRPM6

The TRP superfamily includes over two-dozen channels that are expressed widely and among them conduct both monovalent and divalent cations. Six subfamilies have been defined, of which the melastatin-related TRPM subgroup is one. TRPM channels contain 6 transmembrane helices and probably function as tetrameric complexes. The *TRPM6* gene comprises 39 exons and encodes a protein of up to 2022 amino acids. In addition, and uniquely among the eight members of the TRPM family, TRPM6 and the closely related TRPM7 have an alpha-kinase domain. The kinase function appears not to be necessary for channel function, but coupling between kinase and channel domains may be important in the regulation of channel activity by magnesium.[3]

TRPM6 is expressed in the small intestine and the kidney, and mediates transcellular magnesium transport in both organs. Impaired intestinal magnesium absorption in HSH represents a defect in the active transcellular component.[3] TRPM6 is expressed on the brush border of intestinal epithelial cells.[63] Mg transport through this channel is driven by its electrochemical gradient. Dietary magnesiumrestriction increases renal expression of mRNA for TRMP6.[64] The mechanism of magnesium exit through the basolateral surface is unknown.

In the kidney, TRPM6 expression is greatest in the DCTs, the site of the final modulation of urinary magnesium excretion via transcellular magnesium transport.[65] Its expression is increased when dietary magnesium is restricted, as one would expect. TRMP mRNA levels are increased in response to 17β-estradiol, but not in response to 1,25-dihydroxyvitamin D or parathyroid

hormone.[64] While some have reported that hypomagnesemia in HSH is entirely the consequence of impaired intestinal absorption,[3] others have documented inappropriate renal magnesium wasting in this disease.[54]

Another member of this protein family, TRPM7, shares about 50% homology with TRPM6. It is expressed ubiquitously in tissues, and is proposed to mediate magnesium uptake into cells. Unlike TRPM6, which is confined to the cells of the DCT and collecting duct, TRPM7 is expressed throughout the nephron. In vitro, TRPM6 appears to require the presence of TRPM7 to form functional heteromultimeric channels targeted to the cell membrane.[58] In the N-terminal cytoplasmic domain of these two proteins, disease-causing mutation in TRPM6 or creation of the homologous mutation in TRPM7 prevents the formation of functional ion channels in the cell membrane,[58] emphasizing the potential importance of this interaction in the function of the channel.

A regulatory protein for the alpha-kinase domain of TRPM6 has been identified, the receptor for activated C-kinase (RACK1). RACK1 is expressed in DCT with TRPM6 and appears to inhibit cation channel activity. Experimental reduction in RACK1 levels increases TRPM6 currents. One particular threonine residue (Thr1851) in the alpha-kinase domain of TRPM6 is autophosphorylated and its phosphorylation state appears to be important in the regulation of the ion channel by magnesium and inhibition of its activity by RACK1.[66] Disease-causing mutations in TRPM7 or RACK1 have not been reported.

3.2 Animal Model

Two groups have produced *Trpm6* knockout mice.[67,68] Homozygous deletion of *Trpm6* was lethal at the embryonic stage. In both studies, mice heterozygous for the deletion ($Trpm6^{+/-}$) had significantly lower serum magnesium levels than their normal ($Trpm6^{+/+}$) littermates. In one of the studies, supplementation of magnesium in the dam during pregnancy allowed a few of the homozygous knockout $Trpm6^{-/-}$ mice to survive to term, and those mice exhibited neural tube defects combining exencephaly and spina bifida occulta. Since expression of *Trpm6* is minimal in mouse brain, the mechanism of these defects is not understood.[67]

3.3 Treatment Considerations

Symptoms of hypomagnesemia do not occur at birth, as magnesium is readily transported across the placenta; clinical deficiency becomes manifest after several weeks or months of life.[65] Since permanent neurologic damage can occur if the mineral defects are not addressed, infants with seizures, tetany, or neuromuscular spasms should be treated urgently and aggressively, and at least initially parenterally. Magnesium replacement will promptly raise serum calcium levels, as PTH is released promptly, and end-organ responsiveness to PTH is restored, as magnesium deficiency is corrected. The need for magnesium supplementation is permanent, and dose requirements are very high. Very large oral doses of magnesium salts can achieve adequate (though rarely normal) levels of serum minerals,[46] as the paracellular mechanism of intestinal magnesium absorption remains intact in HSH and paracellular absorption is a linear function of luminal magnesium concentrations.[3] However, large oral doses often induce diarrhea, and deters patient adherence to therapy. Long-term parenteral administration is an alternative.[46] Continuous nocturnal nasogastric magnesium infusion has been shown to be effective with less stimulation of gastrointestinal motility.[47]

4 ISOLATED AUTOSOMAL RECESSIVE HYPOMAGNESEMIA (IRH)

The observation of an increased fractional magnesium excretion in the context of severe hypomagnesemia in two mentally retarded sisters of a consanguineous union has identified IRH as a unique disorder of renal magnesium wasting.[69] Other clinical features included seizures, and laboratory evaluation has demonstrated normal serum and urine calcium. Investigation included homozygosity mapping which showed evidence for linkage to chromosome 4 between markers D4S2623 and D4S1575. The EGF gene is in this chromosome segment and sequencing in the affected individuals identified the homozygous mutation C3209T in exon 22, which cosegregated with the disorder and was absent in 126 ethnically matched control chromosomes. The mutation caused the substitution of the highly conserved proline 1070 within the cytoplasmic tail of EGF by leucine (P1070L).[10]

4.1 Role of Epidermal Growth Factor

The *EGF* gene consists of 24 exons encoding a large type I membrane–anchored precursor protein (pro-EGF) which is proteolytically cleaved and released to generate the 53–amino acid peptide hormone EGF.[70] EGF is expressed in several organs, including gastrointestinal tract, respiratory tract, and kidney. In kidney, EGF localizes to the basolateral membrane of DCT epithelium, as does the EGF receptor.[71,72] Expression of the mutant EGF gene in MDCK cells disrupts protein sorting to the basolateral membrane;[10] in turn, EGF binding to its receptor regulates the apical magnesium channel TRPM6 and failure of proper EGF expression produces DCT magnesium wasting and hypomagnesemia.[10]

This mechanism is supported by the observation of renal magnesium wasting and hypomagnesemia in patients with metastatic colon cancer treated with cetuximab, a monoclonal antibody to the EGF receptor,[73] and in studies of the tyrosine kinase inhibitor erlotinib. Cetuximab blocks binding of EGF to the receptor, halting subsequent signal transduction mediated via the receptor-associated tyrosine kinase.[74] Activation of tyrosine kinase and other downstream proteins in the signal transduction cascade are important in TRPM6 activation,[74] and incubation in vitro of cetuximab with DCT cells and erlotinib with HEK293 cells inhibits TRPM6[75] activation, likely by inhibiting mobilization of TRPM6 from storage vesicles to plasma membrane.[10]

5 AUTOSOMAL DOMINANT RENAL HYPOMAGNESEMIA WITH HYPOCALCIURIA

A syndrome of renal hypomagnesemia with hypocalciuria (HOMG2; OMIM 154020) with autosomal dominant inheritance was first described by Geven et al. in 1987 in two apparently unrelated Dutch families.[76] The index cases in each kindred presented with seizures and were noted to have marked hypomagnesemia. Examination of other family members revealed histories of tremors, tetany, seizures, cardiac arrhythmias, and chondrocalcinosis. Patients did not have hypokalemia or metabolic alkalosis. Gene mapping showed linkage to chromosome 11q23 and screening of candidate genes in both families revealed the identical heterozygous missense mutation, 121G to A, which results in the substitution of arginine for glycine at residue 41 of the FXYD2 protein (G41R).[13,76,77] More recently, two additional families, one Dutch (2 generations) the other Belgian (3 generations), have been reported with the G41R mutation of the FXYD2 gene.[78] Affected individuals had marked hypomagnesemia and hypocalciuria; all patients reported chronic fatigue and tetany with seizures, tremors, and chondrocalcinosis variably reported. Mild hypokalemia and metabolic alkalosis were reported in a minority of subjects and one had stage 3 chronic kidney disease.[78]

5.1 FXYD2

The *FXYD2* gene encodes the γ-subunit of the basolateral Na^+/K^+-ATPase.[13,79,80] The Na^+/K^+-ATPase is responsible for the exchange of sodium moving out of the cell for potassium moving intracellularly and maintenance of transmembrane potential. It consists of a catalytic α-subunit, a chaperone β-subunit, and a third nonobligatory γ-subunit. The γ-subunit is one of seven mammalian members of the FXYD family[81] and expressed predominantly in the kidney, particularly the DCT but not the TAL. The γ subunit influences the Na^+/K^+-ATPase's cation affinities, for potassium in a membrane potential-dependent manner[79,82] and for sodium by reduction of apparent affinity.[79,82] It also regulates the apparent affinity for ATP.[83,84]

Studies in *Fxyd2* knockout mice reveal normal serum magnesium values; however, 24-h urinary magnesium excretion is 47% greater than wild type controls.[85] Interestingly, mice with one active allele had urinary magnesium excretion similar to knockout animals, though again normal serum magnesium, similar to previous findings in mice with a single *fxyd2*.[13] Thus, reduced FXYD2 expression may produce suboptimal renal magnesium handling, but not the marked hypomagnesemia seen in patients with G41R mutation.

In fact, a change in function has been proposed with altered association between the mutant γ-subunit and the Na^+/K^+-ATPase destabilizing the enzyme complex[77,79,80] with depolarization of the membrane potential of DCT cells and increase in intracellular sodium content[13] leading to reduced magnesium reabsorption through the apical TRPM6 channel. In addition, Sha et al. have shown that FXYD2 can mediate basolateral extrusion of magnesium from cultured renal epithelial (MDCK) cells. In MDCK cells, wild-type FXYD2 leads to an increase in transepithelial current in the presence of a large Mg^{2+} concentration gradient across the epithelium. This current is significantly reduced by cotransfection with the FXYD2-G41R mutant.[86]

It remains uncertain how a heterozygous mutation in the *FXYD2* gene causes a dominant phenotype. Expression studies in different cell models (*Xenopus laevis* oocytes and HeLa cells) have revealed mistrafficking of the mutated γ-subunit to a perinuclear location.[13,80] In turn, studies have demonstrated oligomerization of the γ-subunit, and the continued ability of the FXYD2 (G41R) protein to oligomerize with wild type protein.[13,87] This mistrafficking of wild type protein may explain dominant transmission.

5.2 HNF1B/PCBD1

HNF1B (hepatocyte nuclear factor 1B) is a transcription factor involved in embryonic development and tissue-specific gene expression in several organs, including tubular epithelial cells along all nephron segments.[88] HNF1B regulates expression of genes important to renal tubular development and mutations in HNF1B have been observed in 10%–30% of infants noted to have congenital anomalies of the kidney and urinary tract,[89] often cystic dysplasia or a glomerulocystic variant of polycystic kidney disease, with autosomal dominant transmission and high phenotypic variability and incomplete

penetrance. In addition, 25%–75% of persons with heterozygous HNF1B mutations also manifest renal magnesium wasting with hypomagnesemia and hypocalciuria.[90,91] Although hypomagnesemia can be severe and the presenting manifestation of disease in this circumstance,[92] in most patients serum magnesium is only mildly decreased.[90]

The FXYD2 protein contains several HNF1B-binding sites,[90] and HNF1B also regulates the transcription of the FXYD2 protein in vitro.[91] In addition, incubation of HNF1B mutants with wild type protein demonstrates a dominant negative effect on FXYD2 transcription with markedly reduced expression.

HNF1B forms heterotetrameric complexes with the protein pterin-4α-carbinolamine dehydratase/dimerization cofactor (PCBD1).[93] PCBD1 is expressed in distal collecting duct, and expression is increased in mice fed low magnesium diets. PCBD1 binding to HNF1B increases FXYD2 transcription by approximately 50% compared to HNF1B alone.[93] Thus, PCBD1 appears to provide fine-tuning to renal magnesium handling. Studies in patients with homozygous mutations of the PCBD1 gene show mildly decreased or low normal serum magnesium with evidence for urinary magnesium wasting.[93]

6 GITELMAN SYNDROME

GS (OMIM 263800) is an autosomal recessive salt-losing renal tubulopathy that is characterized by hypomagnesemia, hypocalciuria, and secondary aldosteronism, with consequent hypokalemia and metabolic alkalosis.[94] The hypomagnesemia is often severe, and tetany and seizures are commonly the presenting features of the disease.

6.1 Molecular Defect and Genetics in Gitelman Syndrome: SLC12A3 and NCCT

GS is inherited as a recessive trait; heterozygous relatives of GS patients are asymptomatic, and gene mutations are common, with the prevalence of heterozygotes in Swedish and Italian populations estimated at 1%.[95] In 1996, Simon et al. reported that loss-of-function mutations in the *SLC12A3* gene at chromosome 16q13 were associated with GS.[12] *SLC12A3* encodes for the thiazide-sensitive Na^+-Cl^- cotransporter (NCCT). Almost 180 presumed loss-of-function SLC12A3 mutations, including missense, nonsense, frame-shift, splice-site, and deep intronic mutations, have been identified.[96] Genetic heterogeneity has not been observed. The phenotype of GS closely resembles the clinical consequences of therapy with thiazide diuretics, which act by inhibiting NCCT.

6.2 Pathophysiology

Loss-of-function mutations in NCCT impair NaCl reabsorption in the DCT, resulting in increased sodium delivery to the collecting duct and extracellular volume depletion.[97,98] This activates the renin-angiotensin-aldosterone axis.[94] The combined effects of aldosterone and increased sodium delivery to the collecting ducts lead to increased activity of the apical epithelial Na channel (ENaC);[99] reabsorption of sodium through ENaC produces a lumen-negative electrical potential which provides the driving force for increased potassium and hydrogen ion secretion.[100,101] Thus, patients with GS have hypokalemia and metabolic alkalosis. However, unlike patients with Bartter syndrome, the salt wasting and volume depletion in GS are mild.[102] Studies in NCCT-deficient mice have demonstrated lack of apical epithelial sodium channels in the early DCT but marked epithelial hypertrophy and an apical abundance of sodium channels in the connecting tubule.[103] This structural remodeling may limit salt wasting in GS, and in fact NCCT-deficient mice do not show any signs of hypovolemia on a standard sodium diet, but a lower blood pressure after 2 weeks of dietary sodium restriction.[104]

Insights into the mechanisms of hypocalciuria and hypomagnesemia have come from studies of NCCT inhibition by thiazides[105] and of NCCT-deficient mice.[103] The effects of thiazides on excretion of calcium and magnesium are quite different. Thiazides reduce calcium excretion acutely and chronically; in contrast, thiazides increase urinary magnesium loss, and this effect is largely only with sustained therapy.[105] Volume-depletion itself leads to enhanced reabsorption of calcium in the proximal tubule. There is evidence for enhanced calcium reabsorption in the DCT when NCCT is inactivated. The reduced influx of sodium and chloride in the early DCT cells in combination with continued exit of intracellular chloride through basolateral chloride channels causes hyperpolarization of the cell. This may increase calcium entry via apical calcium channels, and studies in NCCT-deficient mice have demonstrated upregulation of these channels.[103] The subsequent increase in intracellular calcium would stimulate calcium efflux via the basolateral Na^+/Ca^{2+} exchanger and the Ca^{2+}-ATPase. However, Nijenhuis et al. have reported that the hypocalciuric effect of thiazides occurred even in mice in whom the Trpv5 calcium channel, through which active Ca reabsorption occurs, is inactivated, and that thiazides upregulate expression of the proximal tubular Na/H exchanger but not of proteins involved in DCT calcium transport.[106]

In mice treated chronically with thiazides, or in whom the *NCC* gene was knocked out, expression of the magnesium channel Trpm6 in DCT was significantly depressed. Further, *NCC* knockout mice manifest atrophy of DCT cells reducing the mass of epithelial cells for active magnesium transport.[106] Either or both of these effects could

explain urinary magnesium loss in GS. Another factor might be a potential inhibitory effect of aldosterone on magnesium reabsorption.[105]

6.3 Phenotype Variability and Clinical Features

GS is usually detected in adolescents or young adults; only 2 of 16 patients reported by Bettinelli et al. were diagnosed before 6 years of age.[97] Though salt craving and polydipsia are often reported, patients with free access to dietary sodium rarely demonstrate clinical volume depletion.[107] However, diagnosis at a young age will sometimes be made in the context of a gastrointestinal illness. Children with GS will be prone to early volume depletion in this circumstance and evaluation during such an episode may reveal severe hypokalemia and metabolic alkalosis.[97]

Most GS patients are diagnosed as a result of symptoms related to hypomagnesemia, which can be moderate to severe: serum magnesium concentrations are often less than 1.0 mg/dL (0.41 mmol/L).[97,107] As discussed previously, severe hypomagnesemia blocks parathyroid hormone secretion and impairs its bone resorbing activity. Furthermore, metabolic alkalosis increases protein binding of ionized calcium. Patients may experience tetany, paresthesias, even seizures. Interestingly, cardinal features, such as hypocalciuria and hypomagnesemia, might change during the life cycle of a given patient, reflecting dietary changes or compensatory mechanisms.[102]

6.4 Treatment of Gitelman Syndrome

Correction of hypokalemia often requires large amounts of potassium chloride, up to 10 meq/kg/day in children, divided three or more times daily.[108] Treatment with chloride reduces potassium and hydrogen ion secretion by reducing luminal electronegativity[100,101] and improvement in metabolic alkalosis decreases symptoms related to hypomagnesemia. However, potassium chloride therapy is often limited by nausea.

Similarly, treatment of hypomagnesemia to achieve near normal serum magnesium and prevent tetany often requires oral magnesium supplements, generally magnesium oxide or magnesium chloride, at doses that can induce diarrhea and at dosing intervals that make compliance difficult.[107] In this context, treatment with spironolactone may be helpful. Colussi et al. reported that spironolactone reduces urinary potassium and magnesium excretion in GS patients, lowering the amount of potassium and magnesium supplements required.[108] In that same vein, treatment with angiotensin-converting enzyme inhibitors or angiotensin-receptor blockers may prove beneficial, but as with spironolactone, monitoring for hypotension in this clinical setting is particularly important.

7 HYPOMAGNESEMIA IN BARTTER SYNDROME

Hypomagnesemia is less common, and when present less severe, in Bartter syndrome than in GS. Unlike GS, Bartter syndrome is genetically heterogeneous, with at least five genes each accounting for a subgroup of patients. Bartter syndrome type III (OMIM 607364), in which the basolateral chloride channel CLC-Kb is dysfunctional, can present with a phenotype that overlaps with that of GS, particularly after early childhood, and includes hypomagnesemia more commonly than other subgroups of Bartter syndrome.[109–111] Another genetic causes of Bartter syndrome is activating mutation in the calcium-sensing receptor (CaSR) (Bartter type V), in which mild depression of serum magnesium levels can occur.[8]

8 AUTOSOMAL DOMINANT HYPOCALCEMIA

Constitutive activating mutations in the CaSR are also associated with autosomal dominant hypoparathyroidism (ADH, OMIM 146200), in which nearly half of patients are hypomagnesemic.[112] Factors contributing to hypomagnesemia when the CaSR is persistently activated include suppression by the CaSR of parathyroid hormone release and inhibition by the CaSR of the potassium channel ROMK in the TAL, with consequent reduction in the lumen-positive potential difference that drives cation transport in that nephron segment.[113]

Therapy with vitamin D or calcium in ADH can increase urinary calcium excretion dramatically even though serum calcium levels may not rise above the low-normal range.[112] This may lead to polyuria, nephrocalcinosis, stones, and even irreversible renal dysfunction. Thus, treatment of hypocalcemia in ADH should be used only in symptomatic patients.

9 SEIZURES, SENSORINEURAL DEAFNESS, ATAXIA, MENTAL RETARDATION, AND ELECTROLYTE IMBALANCE (SeSAME SYNDROME)/ EPILEPSY, ATAXIA, SENSORINEURAL DEAFNESS, AND TUBULOPATHY (EAST SYNDROME)

In 2009, Scholl et al. and Bockenhauer et al. separately reported an autosomal recessive disease isolated to chromosome 1q23.2 and caused by mutations in *KCNJ10*, a gene encoding the Kir4.1 potassium channel.[14,15] Clinical manifestations included neonatal seizures, ataxia, developmental delay, sensorineural deafness, and defects

in electrolyte regulation, including hypokalemia, metabolic alkalosis, and hypomagnesemia. The respective authors used these clinical features to coin the acronyms SeSAME syndrome[14] and EAST syndrome.[15]

9.1 Role of Kir4.1 in Kidney

In the DCT, basolateral Na^+/K^+-ATPase transports sodium to the interstitium and potassium to the intracellular compartment against chemical gradients. Basolateral potassium channels recycle potassium to the interstitium[114] and Kir4.1 is the dominant channel in DCT.[115] This ensures adequate extracellular potassium as substrate for optimal function of the Na^+/K^+-ATPase, which maintains intracellular sodium at low levels; in the DCT this mechanism provides the driving force for Na and Cl influx to the cell through NCCT, the channel mutated in GS.[83] Basolateral potassium efflux through Kir4.1 also maintains a negative intracellular electrical potential. In turn, depolarization in the DCT inhibits STE20-related proline-alanine-rich kinase (SPAK) expression. SPAK regulates NCCT phosphorylation and expression.[115] Thus, loss of Kir4.1 function would be predicted to produce a pathophysiologic sequence similar to GS, with DCT salt wasting and volume depletion, secondary activation of renin-angiotensin-aldosterone activity, and resultant hypokalemia and metabolic alkalosis. Indeed, these abnormalities are seen in patients with *KCNJ10* mutation,[14,15] and excessive urine sodium excretion has been demonstrated in *Kcnj10* knockout mice.[15] The mechanism for hypomagnesemia would presumably be the same as that in GS, discussed previously.

Kir4.1 is also expressed in cochlea and central nervous system. In the ear, Kir4.1 is important in the generation of endocochlear potential,[116] and *Kcnj10* knockout mice are markedly hearing-impaired.[15] In the brain, Kir4.1 localizes to glial cells of the cerebral cortex and cerebellum and likely maintains neuronal resting membrane potential through a process known as potassium spatial buffering.[117,118] Abrogation of Kir4.1 activity may enhance neuronal excitation and predispose to seizures. In addition, *Kcnj10* knockout mice have dysmyelination of the spinal cord and brainstem; the knockout mice are growth retarded compared to heterozygous littermates, demonstrate lower extremity weakness and ataxia, and expire in the days after birth in the context of seizures and these central nervous system dysfunctions.[15]

9.2 Genetics and Clinical Features

Using a genome-wide analysis of linkage followed by identification of *KCNJ10* as a candidate gene in the region of interest, patients have demonstrated missense or premature termination mutations.[14,15] These mutations are at positions completely conserved in all vertebrate species evaluated.[14] In addition, Scholl et al. demonstrated that two mutations involved positions also conserved in the potassium channel ROMK and that ROMK mutations at these points abolish ROMK function and produce the Bartter syndrome phenotype.[15]

Neurologic and developmental abnormalities have been the dominant clinical features in the patients reported. All have demonstrated generalized seizures in the first 6 months of life and all have shown delay in development of speech and motor functions. Similar to the knockout mice, motor strength was especially marked in the lower extremities. As they learned to walk, all patients demonstrate ataxia. Interestingly, brain MRI showed no evidence for demylenation in the patients evaluated.[15] All patients demonstrate sensorineural hearing loss with progression toward deafness.

Though the metabolic abnormalities do not generally produce dramatic symptoms, the findings are diagnostically important. Hypokalemia and metabolic alkalosis in the context of the central nervous system and hearing abnormalities distinguish SeSAME/EAST syndrome from other neurodevelopmental diseases. Like patients with GS, studies in these patients show urinary sodium wasting, elevated renin and aldosterone, and blood pressure at the low end of the normal range. Also similar to GS, patients show hypocalciuria and hypomagnesemia in the context of elevated fractional excretion of magnesium.[14,15]

However, unlike GS, the hypomagnesemia in SeSAME/EAST syndrome is mild and tetany has not been reported, though the profound neurologic symptoms patients demonstrate, including tremor, may be exacerbated by hypomagnesemia. Patients are often treated with magnesium supplements but doses required are lower than those usually necessary in GS.[15] In contrast, SeSAME/EAST syndrome patients may require potassium chloride supplements at doses similar to those in GS; given the pathophysiology, spironolactone may prove helpful.[108]

10 KCNA1/Kv1.1 MUTATION AND ITS ASSOCIATION WITH ISOLATED HYPOMAGNESEMIA

Glaudemans et al. have reported a large Brazilian family with isolated hypomagnesemia with autosomal dominant inheritance.[9] Affected individuals have recurrent episodes of tetany, tremors, and muscle weakness, often beginning in infancy. Moderate to severe hypomagnesemia [<0.40 mmol/L (<0.8 meq/L); normal

0.70–0.95 mmol/L (1.4–1.9 meq/L)] has been demonstrated between and during symptomatic attacks, and symptoms have improved with magnesium replacement therapy. Urinary magnesium excretion is not diminished despite the hypomagnesemia, indicating an abnormality in renal magnesium handling. However, unlike most other disorders of renal magnesium homeostasis, the finding is isolated; affected individuals demonstrate normal urine and serum calcium and normal serum potassium.[9]

The disorder has been linked to chromosome 12p13 and a specific mutation in the *KCNA1* gene, which encodes the voltage gated potassium channel Kv1.1. This heterozygous *KCNA1* A763G mutation causes substitution of the highly conserved neutral asparagine at position 255 for the acidic aspartic acid (N255D) in Kv1.1.[119] This mutation is in the third transmembrane segment of the Kv1.1 protein close to the voltage sensor and may destabilize the channel structure and trafficking.[9]

10.1 Mechanism for the Associated Hypomagnesemia

In kidney, the Kv1.1 channel is seen in abundance along the DCT luminal membrane along with TRPM6. Patch clamp experiments have shown that wild type Kv1.1 is important in establishing the negative potential across the DCT luminal membrane and seems to provide the driving force for TRPM6 magnesium reabsorption. With expression of mutated N255 in Kv1.1, negative membrane potential is diminished; this depolarization appears to decrease magnesium influx. The disorder is inherited in an autosomal dominant manner and mutations are heterozygous. In fact, expression of mutated Kv1.1 does not affect the expression wild-type Kv1.1 channels at the plasma membrane.[9] However, Kv1.1 channel subunits can assemble with other Kv channel subunits to form heterotetramers[120]; affected individuals likely express heterotetrameric Kv1.1 channels composed of wild-type and mutated subunits, with the N255 mutant exerting a dominant negative effect on overall channel activity and voltage.[9]

Mutations in *KCNA1* are also known to be associated with episodic ataxia type 1, a rare autosomal dominant neurological disorder presumably caused by defective Kv1.1 in the cerebellum.[121] Episodic ataxias are characterized by spells of dyscoordination and imbalance, often with associated progressive ataxia; however, hypomagnesemia has not been reported in these patients. The different phenotype for these two disorders may be explained by a difference in the composition of Kv1.1 in the brain compared to the kidney, with possible variation in assembly and interactions with other Kv1 units which may be tissue-specific.[122,123]

11 HYPOMAGNESAEMIA WITH MITOCHONDRIAL INHERITANCE

Wilson et al. described a kindred identified through a proband with symptomatic hypomagnesemia in which family members exhibit a high prevalence of hypomagnesemia, hypertension, and hypercholesterolemia.[124] Hypomagnesemia in this kindred was associated with excessive fractional magnesium excretion, and thus resulted from impaired magnesium reabsorption. All 32 hypomagnesemic family members were on the same maternal lineage, and none of the hypomagnesemic men transmitted it to their offspring. Urinary calcium excretion was reduced in subjects in the maternal lineage, consistent with a defect in the DCT. Serum calcium levels were normal. Subjects also had hypokalemia with excessive potassium excretion.

Adults in this maternal lineage had hypertension, which was age-dependent, and hypercholesterolemia, with increased LDL and VLDL. These differences from those family members in the nonmaternal lineage were significant even though many were on treatment. These findings suggested mitochondrial inheritance, and this was supported by a genome-wide linkage study that showed no evidence of linkage to any region of the nuclear genome. Direct sequencing of the entire mitochondrial genome in this family revealed 14 sequence variants, 13 of which were known polymorphisms. The one novel variant was a thymidine-to-cytidine transition at nucleotide 4291, within the mitochondrial $tRNA^{Ile}$ gene and immediately 5′ to the $tRNA^{Ile}$ anticodon. This mutation had not been reported in the thousands of sequences recorded until then in the Human Mitochondrial Genome Database, was absent among 170 control individuals, and was not found in any of the family members in the nonmaternal lineage. It results in an unstable stem-loop in the isoleucine tRNA that impairs ribosome binding.

Magnesium reabsorption in the DCT is driven by ATP-dependent Na reabsorption. DCT cells are rich in mitochondria and have the highest energy consumption of any nephron segment.[125] Clustering of hypertension and hypercholesterolemia with hypomagnesemia in the maternal lineage indicates that they too are consequences of mitochondrial dysfunction. The prevalence of other phenotypes associated with mitochondrial dysfunction, including migraine headaches, sensorineural hearing loss, and hypertrophic cardiomyopathy, was also increased on the maternal lineage in this family. Muscle biopsy in one affected family member revealed characteristic features of mitochondrial dysfunction, and in vivo NMR spectroscopy showed evidence of reduced ATP production.

Other evidence has implicated mitochondrial dysfunction with insulin resistance, obesity, and

hypertriglyceridemia, traits which were absent in this kindred. But the clustering of hypertension and hypercholesterolemia with this mitochondrial mutation, and the known decline in mitochondrial function with age, suggest a role for mitochondria in the metabolic syndrome.

Hypomagnesemia has been reported in individual cases of two recognized mitochondrial myopathies, both with microdeletions of mitochondrial DNA. Pearson syndrome is a multiorgan disorder characterized by exocrine pancreatic dysfunction and vacuolization of marrow hematopoetic precursor cells with sideroblastic anemia, often with diabetes, organic aciduria, and features of the renal Fanconi syndrome. Death often occurs in early childhood with muscle weakness and lactic acidosis. When reported hypomagnesemia has been associated with severe renal magnesium wasting.[126] Kearns–Sayre syndrome comprises progressive ophthalmoplegia, retinopathy, cerebral ataxia, cardiac conduction defects, diabetes, deafness, and cognitive impairment, in which hypomagnesemia has been described in some cases. Mutations in Kearns–Sayre can be in the mitochondrial or nuclear DNA.[127]

An additional syndrome of mitochondrial cytopathy, HUPRA syndrome, results from mutations in *SARS2*, a nuclear gene at 19q13.2 encoding an aminoacyl-tRNA synthetase, that functions in mitochondria. The acronym represents the features of hyperuricemia, pulmonary hypertension, renal failure, and alkalosis. It was described in three infants from one Palestinian village including two in consanguineous kindred. All three had significant hypomagnesemia with inappropriate renal wasting, and all died in the first months of life.[128]

12 CNNM2 MUTATIONS IN DOMINANT HYPOMAGNESEMIA

Mutations in a novel gene, *CNNM2*, have been reported in association with hypomagnesemic syndromes with either dominant or recessive inheritance. The first were reported by Stuiver et al.[129] in two hypomagnesemic families. The gene was identified as a candidate because it was among those found to be differentially expressed in *Cldn16*-deficient mice. It was of interest because it had previously been shown to be upregulated in magnesium deficiency and to mediate the transport of magnesium and a number of other divalent cations but not calcium.[130] The investigators studied two families with symptomatic hypomagnesemia with apparent dominant inheritance, one of which had previously been reported.[131] Symptoms included atypical seizure-like events, muscle weakness and headaches, and resolved with magnesium replacement. Hypomagnesemia was accompanied by inappropriate urinary magnesium loss. Serum calcium levels were in the low-normal range, other serum electrolytes normal, urinary calcium normal, and PTH levels low. Mutation in *FXYD2, EGF*, and *SLC12A3* were excluded by mutation analysis.[129]

Heterozygous mutations were identified in these two families, one a deletion of a single base pair leading to frameshift and truncation of the protein, and the other a missense mutation in a highly conserved domain. These mutations segregated with the disease and were not present in 200 ethnically matched control chromosomes.[129]

The same group has now reported recessive inheritance of symptomatic hypomagnesemia with seizures and mental developmental delay in five families.[132] Patients exhibited severe intellectual disability with impaired speech and motor skills. Mutations were all missense and predicted to alter protein function. The two patients with homozygous mutations presented in the first days of life and had structural brain malformations and much more severe symptoms than the remainder, who were heterozygous and presented after months or years.

CNNM2 is expressed in human kidney, brain, and lung. Knockdown of *cnnm2* isoforms in zebrafish produces abnormal brain development and neurological function that was rescued by wild type but not by mutant *Cnnm2* cRNA.

CNNM2 encodes cyclin M2, which in the kidney is expressed on the basolateral surface of cells of both the TALH and DCT in human specimens. Cyclin M2 expression is induced by magnesium depletion in mouse DCT cells[129] and in a cultured lymphoblastic cell line.[11] Transfection of DCT or HEK293 cells with wild-type *CNNM2* increased magnesium transport, in contrast to transfection with the disease-associated missense.[129,132]

When expressed in the kidney HEK293 cell line cyclin M2 does not transport magnesium, but both isomorphs studied form complexes with a range of intracellular proteins suggesting a role in regulation of magnesium transport.[11] In any case, its nephron localization, modulation of expression by magnesium and mutation associated with symptomatic hypomagnesemia clearly indicate that it plays an important role in magnesium reabsorption. This view is strengthened by a large genome-wide association study demonstrating significant association of *CNNM2* variants with serum magnesium levels.[133]

An additional (American) family has been reported with isolated autosomal hypomagnesemia in which linkage to the *FXYD2* locus on chromosome 11q23 was excluded.[134] Whether this might represent mutation in *CNNM2*, or perhaps yet another gene yet to be identified remains to be seen.

13 TREATMENT AND COUNSELING

Treatment considerations specific to a particular disease associated with renal magnesium wasting are noted in the sections aforementioned. In general, patients with symptoms that can be related to hypomagnesemia, such as tetany, tremor or seizures should receive magnesium supplements. However, given the underlying nature of these diseases, achieving a serum magnesium concentration within the normal range is usually difficult. Because urinary magnesium wasting is ongoing, and supplementation at high or even moderate oral doses carries the risk of diarrhea, optimal therapy requires dosing magnesium supplements multiple times per day, which can make compliance difficult.

In certain diseases, such as SeSAME/EAST syndrome, diagnosis is usually made based on characteristics unrelated to renal magnesium wasting, and the hypomagnesemia may be mild. In fact, asymptomatic mild hypomagnesemia is relatively common in the general population; it is estimated to occur in 2.5%–15% of adults.[135–137] Increasing epidemiologic and experimental data indicate that even mild hypomagnesemia may be associated with various health risks. Induction of hypomagnesemia in individuals without diabetes reduces insulin sensitivity[138] and an inverse relationship between serum magnesium concentrations and glycemic control in patients with type 2 diabetes has been reported.[139,140] Thus, hypomagnesemia may increase risk of developing type 2 diabetes and it may increase the risk of developing complications such as retinopathy[141] and nephropathy.[142] An inverse relationship between baseline serum magnesium concentrations and development of coronary artery disease has been reported in a large epidemiologic study[143] and experimental data indicate that magnesium depletion initiates a cascade of inflammatory and oxidative events that promotes cardiac dysfunction in experimental animals.[144]

Ultimately, therapy that corrects the renal magnesium wasting would be ideal. Angiotensin-converting enzyme inhibitors or angiotensin-receptor blockers may reduce glomerular filtration rate and magnesium delivery to the distal nephron. In practice, however, their effectiveness is limited. Otherwise, magnesium supplements, generally magnesium chloride, or oxide, at the greatest doses and dosing intervals that can be tolerated, remain the mainstay of therapy.

If a clinical diagnosis of a specific inherited renal magnesium-wasting disorder has been made the patient and family should be counseled as to the apparent inheritance pattern and the likelihood of future affected offspring. This may include consultation with a genetic counseling professional. Genetic testing may be available for certain diseases; current information as to the status of such testing can be facilitated at www.genetests.org. Depending upon the certainty of the diagnosis and family considerations, diagnostic testing, including prenatal diagnosis, may be pursued.

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CHAPTER

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Genetic Hypercalciuria: A Major Risk Factor in Kidney Stones

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1 INTRODUCTION

Kidney stones are prevalent, cause considerable morbidity though little mortality. The most common metabolic abnormality in patients with kidney stones is hypercalciuria, which is a complex metabolic trait that is dependent on three major organs: the amount of dietary calcium absorbed, any net calcium released from bone resorption in excess of formation, and the extent to which filtered calcium is reabsorbed in the renal tubule. Each of these calcium fluxes is under control of number of factors and hormones, including parathyroid hormone (PTH) and 1,25-dihydroxy-vitamin D. Whether a patient forms a kidney stone is dependent not only on the magnitude of the hypercalciuria but also on urinary volume, excretion of other ions, including oxalate, citrate, and phosphate, and on local factors in the urinary tract. Hypercalciuria, and resultant stone formation, is a partially inherited trait. Given the many determinants of not only urine calcium excretion, but also the other factors that determine whether a patient will form a kidney stone, it is clear that multiple genetic loci are involved. In this chapter, we will describe the progress made in understanding the genetic basis for hypercalciuria and stone formation in both experimental models and in man.

2 CLINICAL DESCRIPTION AND DEFINITION

The overall lifetime risk of kidney stones in the world is 10%–20%, which poses a formidable burden on health and health care cost. Eighty percent of kidney stones are primarily calcium in composition.[1–6] Although a definitive etiology is unveiled only in a fraction of patients with calcareous kidney stones, one can usually identify empiric risk factors in urine chemistry that confers the stone forming propensity, and when corrected empirically lowers the risk of formation, though not a cure,[7] One of the most prevalent and most important risk factor for kidney stones is hypercalciuria.[1,8] While this may seem logical and even trivial at first glance, the situation is in fact far more complex.

While the kidney can render the urine virtually sodium free, there is a minimal obligatory amount of calcium excreted in urine over a given time. The conservation of water mandates a low urine volume, which imposes a herculean challenge to the kidney to keep calcium from forming insoluble complexes and precipitating. From this vantage point, the definition of hypercalciuria becomes not so straightforward. The practitioner uses highly pragmatic "cut-off" type of definitions such >250 mg (6.25 mmol)/24 h in males or >200 mg (5 mmol)/24 h in females or >4 mg (0.1 mmol)/kg/24 h for both gender for adults and 4 mg/(0.1 mmol) kg/day, or a urinary calcium (uCa)/creatinine ratio higher than 0.2 for children.[9] These are practical guidelines especially for enthusiasts of dichotomous traits, to decisively indicate whether an individual does or does not have hypercalciuria. However, such definitions are is clearly oversimplistic and may even erroneous.

There are very few true dichotomous variables in biomedicine, perhaps life versus death and nongravid versus gravid being prime examples. Rate of uCa excretion is a genuine continuous variable over a wide physiologic range with blurred boundaries so the clinical laboratory's distinct cut-off is in many ways ludicrous. What is an "appropriate" amount of calcium in the urine? Similar to many urinary electrolytes during zero external balance, the excretion rate of substances simply reflect the ingestion rates and as such, the "normal" rate of excretion of

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calcium should be the rate of intake of calcium. In addition to calcium intake, there are other physiologic factors that determine the rate of excretion of calcium; the main ones being dietary sodium,[10] which determines extracellular fluid volume, exogenous and endogenous acid production,[11–13] and perhaps nonacid related components in protein.[14] Thus the "appropriate" amount of uCa needs to be interpreted in the light of these modifiers before one can ascribe pathophysiologic origins to the calciuria.

From the point of view of disease, one is basically considering the ability of calcium in imparting risk of kidney stones so hypercalciuria can be statistically defined as the level above which there is a significant escalation of probability of stone formation.[8] Therefore if one visualizes calciuria in terms of its lithogenic tendency, then other parameters, such as urinary volume and relationship of calcium to other electrolytes also needs to be taken into consideration when assessing calciuria. The two major anions that forms insoluble complexes with calcium and consequently eventuate in the solid phase of the urolith are oxalate and phosphate.[1] Therefore, a particular level of uCa may or may not cross the threshold into the pathophysiologic range depending on the amount of oxalate and phosphate available for complexation. In contrary to the prourolithic anions oxalate and phosphate, citrate chelates calcium in a stable and soluble complex so a given level of uCa may or may not confer stone risk depending on the level of urinary citrate.[15] The use of computer-derived predictors of stone risks instead of absolute rates of uCa partially aids in the interpretation of such complex urine chemistry.

As evident from the above description, the quantitative analysis of uCa excretion is far from simple. Aside from the construal of its magnitude, the origin of hypercalciuria is equally complex. uCa is the composite net result from fluxes stemming from three organs, the intestine, bone, and kidney; each in turn is governed by a wide variety of factors.[1] Thus as a phenotypic trait, calciuria is no less complicated than blood pressure. From the physiologic knowledge of calcium homeostasis, one can deduce that genetic determinants will be distributed over many loci and thus propound at formidable challenge to those who attempt to unravel their identities and mode of action. This chapter will summarize the efforts devoted and successes secured thus far in this field.

3 HUMAN KIDNEY STONES AND HYPERCALCIURIA: A COMPLEX GENETIC TRAIT

3.1 A Formidable Problem

One remarkable feature in this field is the fact that no one has cast any doubt that a significant portion of the propensity to form kidney stones and its major risk factor hypercalciuria, in humans are hereditary in origin. Yet, with the exception of a few rare monogenic causes, we have little idea, and even less so, proof, of any loci in human calcareous nephrolithiasis.[16–18]

Several features inherent to a complex polygenic trait render the *en face* tackling of the genetics of hypercalciuria and kidney stones a formidable task. The ability of large number of nongenetic factors yielding the same phenotype as some genetic determinants (phenocopy) is exceedingly common in hypercalciuric kidney stone formers. Many acquire medical conditions, as well as lifestyle and dietary factors can and frequently masquerade genetic causes of hypercalciuria in urolithiasis.[1] There are multiple loci (polygenic) that determine uCa excretion; each may have incomplete and capricious effects (variable penetrance) resulting in inconsistent genotype–phenotype relationships. Affected individuals may inherit different sets of genes that predispose to hypercalciuria (loci heterogeneity) so collections of different causative and modifying genes may all lead to an identical phenotype. All these confounders in concert constitute a problem of nightmarish proportions for the investigator who strives to identify individual genetic loci for hypercalciuria. In addition, the clinical database often is limited to "kidney stones" as a phenotype with no reference to stone constituent and urinary biochemistry. The enormous heterogeneity of stones in the absence of intermediate phenotype further amplifies this problem to the nth degree. With that in the foreground, one questions if there is any hope of ever unraveling this mystery and translate it to clinical diagnostics and therapeutics? A reassuring answer is that progress is being made on many fronts. A summary will be provided here but the reader is referred to an excellent recent review by Attanasio for a more detailed discussion.[19]

3.2 Familial Aggregation

The familial clustering of kidney stones and hypercalciuria has been documented for decades.[20] Despite the often-cited caveat of how family members also share similar lifestyles, a number of studies have tried to correct for shared home environment and still show an increased risk of kidney stone when a family member is involved. In a large epidemiologic study of more than 3×10^5 patient-years and adjusting for dietary factors, age, and body mass index, the authors found a 2.57-fold higher risk of kidney stones when there is a positive family history.[21] Four independent case-control studies amounting to combined number of cases and control each exceeding 1000, have shown that the relative risk (λ_R) is in the order of two- to fourfold higher, and hereditary contribution (defined as ratio of genetic variance to the total phenotypic variance; h^2) to be 40%–60%, which

is rather remarkable considering how diverse were the source of these studies.[22–25]

In a segregation analysis of 221 stone forming families (567 subjects), hypercalciuria was used as an intermediate phenotype, and found to have a hereditability of about 60% and transmitted in a polygenic fashion.[26] Hypercalciuria seems to have as strong hereditability as kidney stone phenotype. This further supports the paramount importance of hypercalciuria compared to the other risk factors in the pathogenesis of urolithiasis and indicates hypercalciuria as one of the strongest genetic components in urolithiasis.

3.3 Twin Studies

Another powerful design is twin studies, which partially circumvents the environmental contamination by comparing monozygotic to dizygotic twins and/or plain siblings; all reared in virtually identical environments. Three such studies have been performed.[27–29] The report by Goldfarb et al. sampled 179 dizygotic and 202 monozygotic twins from the Vietnam Era Twin Registry and found concordance rate of 32% in monozygotic twins compared to 17% in dizygotic twins; an effect that cannot be explain by the documentable dietary information.[30] Other twin studies that obtained urinary chemistries found hereditability of uCa excretion rate to be ~50%.[28,29]

3.4 Classical Nature versus Nurture

One prominent feature that any practitioner will profess from anecdotal experience is that one very uncommonly encounters kidney stones in subjects of African origin. This was documented in multiple epidemiologic studies repeatedly in North American Blacks who do not have vastly different diets than Caucasians.[31] The frequently cited epidemiologic longitudinal study of Stamatelou et al. that showed increasing prevalence of kidney stones with time also showed persistently lower stone incidence in African Americans in the 1970s through the 1990s suggesting that the difference is inherent in the ancestry.[32] In addition to stone prevalence, a similar difference has been noted in uCa excretion with much lower rates in Blacks.[33,34] In the gap of stone prevalence between American Whites and Blacks reside the intermediate values for American Hispanics and Asians, which speaks strongly for the genetic origin of the predisposition.

The term "stone belt" was coined many decades ago to describe the pronounced regional differences in incidence and prevalence of kidney stones, with particularly high numbers in the Middle East, Southeast Asia, and the Southern United States. Although climate, diet, and lifestyle no doubt all influence this to a major extent, one cannot rule out genetic components. In the city of Toronto with extensive multiethnicity, immigrants from different parts of the world, although do retain a small of their culture and hence part of the diets, have by-and-large adopted the westernized lifestyle. Interestingly, the stone prevalence still reflects their ethnic origin rather than convergence into a common incidence along with their homogenization into the comforts of North American suburbia.[35]

3.5 Candidate Gene Approach

The candidate gene approach was used to target putative loci. Some have demonstrated association of polymorphisms of genes along the vitamin D axis with one form of phenotypic parameter or another.[36–43] However, phenotype studies in vitamin D receptor expression, induction, coding region sequence between controls and hypercalciuric stone formers were negative.[44,45] Candidate genes studies in French Canadian sib-pairs, including the vitamin D receptor, 1-α-hydroxylase, the calcium sensing receptor (CaSR), and crystallization modifiers, such as osteopontin, Tamm–Horsefall protein, and osteocalcin-related gene were not conclusive.[46,47] Association studies in an Italian cohort suggest that a R990G mutation of the CaSR which represents a functional polymorphism,[48] is a possible genetic determinant of renal stone formation,[49] but the pathophysiology is not clear. There were additional data recently in case-control designs that showed different allele frequency for the CaSR in stone formers versus normal although this study took the association proximally to serum and uCa.[50] The pathophysiologic link between the CaSR and lithogenicity is still elusive.

The paracellular protein Claudin-14 also has alleles that are different in frequency between stone formers and controls.[50]

In a Swiss cohort, three nonsynonymous polymorphisms in the intestinal calcium channel TRPPV6 exist in higher frequency in calcium stone formers and interestingly, contemporaneous presence of all three polymorphisms in the calcium channel TRPV6 results in increased channel activity.[51] It is plausible that this intestinal calcium channel can contribute to increased gut absorption.[52] The mutation of the Na-phosphate cotransporter NaPi-2c(SLC34A3) imparts renal phosphate wasting, secondary compensatory high vitamin D, and hypercalciuria.[53] Mutations in the vitamin D degrading enzyme CYP24A1 leads to reduced metabolism via 24-hydroxylaiton and increased 1,25-dihydroxy-vitamin D levels.[54] In 250 unrelated kidney stone formers, biallelic mutations were detected in SLC26A1 (Sat1) in two unrelated individuals with calcium oxalate kidney stones,[55] which is compatible with the hyperoxaluric mice with Sat-1 deletion.[56]

In two case-control studies but large sequencing effort by the same group of investigators, thirty candidates

genes were sequenced in both adults and children with kidney stones.[57,58] Although there are subtle differences between the two studies, the conclusion is a rather surprising over about 15%–15% of stone formers have bases (presumed to be mutations), in 14 of the 30 analyzed genes. This is a rather high prevalence, which awaits analysis of the function of the gene products, the pathophysiologic impact, and the phenotype of the affected individuals. For example, Do all the heterozygous carriers of base changes in the two subunit if the dibasic amino acid transport have cystine or calcium stones?

3.6 Genome-Wide Associations

To date, there has been modest success in genome-wide association studies (GWAS) in humans in terms of identification of loci. A small-scale whole-genome linkage analysis of three kindreds with absorptive hypercalciuria and low bone mineral density (BMD) using nonparametric testing found one locus with a candidate in this region, which is the soluble adenylyl cyclase.[59–61] Polymorphisms in this gene are associated with normal BMD variation in healthy premenopausal women and men adults. There was significant evidence of association with one single-nucleotide polymorphism in both men and women but adjacent single-nucleotide polymorphisms did not corroborate the association.[59] There is a whole host of functions for the soluble adenylyl cyclase protein but one appears to be a mediator of low bicarbonate-induced bone resorption.[62]

A GWAS conducted in >3,700 cases and >42,500 controls from Iceland and the Netherlands found synonymous variants in the Claudin gene that associate with kidney stones.[63] Approximately 62% of the general population is homozygous for this variant and carriers are estimated to have 1.64 times greater risk compared to noncarriers. Claudin14 is a paracellular protein that regulates calcium transport in the renal thick ascending limb. A genome-wide approach in over 9300 European subjects identified common variants of the CLDN14 gene exerting influence on the urinary Mg^{2+}-to-Ca^{2+} ratio in urine.[64]

The same variants were also found to associate with reduced BMD at the hip and spine.[63] From a similar Icelandic and Dutch database, another GWAS found a variant positioned next to the UMOD gene which encodes uromodulin (Tamm–Horsfall protein), and this variant seems to protect against kidney stones.[65] Uromodulin confers protection from kidney stones through yet unknown mechanisms.[66,67]

In another Icelandic GWAS of 5,400 kidney stone cases, including 2,100 cases of recurrent kidney stones and 279,870 controls, they identified 2 rare missense variants of SLC34A1 and the renal calcium channel TRPV5 associating with recurrent kidney stones. There were also some associations with biochemical traits but no model was constructed for pathobiology.[68]

3.7 Summary

Several points are noteworthy from the current body of data. There is no doubt from population-based studies that there is a prominent genetic component to hypercalciuria and kidney stones. Case-control and twin studies have repeatedly and definitely proven that. However, the loci remain elusive after decades of study. It is not true that we are devoid of data. The situation is not unlike hypertension, dyslipidemia, and diabetes mellitus although hypercalciuria and nephrolithiasis have not received the same publicity. As more and more sophisticated robust techniques emerge, more and more loci will surface. It will be difficult to discern the true from false candidates. Unraveling the origins of this complex polygenic trait will be a formidable challenge. Correlation of the candidate gene with gene product function and whole organism physiology and pathophysiology will be the most critical part of this endeavor. The knowledge gain from monogenic diseases and polygenic animal models (*vide infra*) will be valuable in unraveling this mystery particularly when the function and dysfunction of the gene products can be linked to physiology and pathophysiology respectively.

4 MONOGENETIC CAUSES OF HYPERCALCIURIA

When one focuses more on intermediate or endophenotypes (when the circumstances permit), the picture assumes a slightly different flavor. There are clear Mendelian conditions in humans that cause hypercalciuria and predispose the subject to kidney stones. An even larger number of candidate loci have been identified in animal models of gene deletion that results in hypercalciuria. An indepth account of these conditions is beyond the scope and space allowance of this manuscript. These conditions are summarized in Tables 43.1 and 43.2.

The authors will let the tables provide a précis of the conditions and the references. A few specific remarks will be submitted and highlighted in the context of our current topic. The rarity of these human conditions frequently lessens their appeal to practitioners who also question the relevance of single gene deletions experiments in rodent. However, monogenic human diseases and rodent models actually provide very valuable tools to investigate the mechanisms of hypercalciuria.

One powerful feature of these monogenic diseases is the fact that they have singular lesions in one gene product that allows one to trace and pathophysiologic pathway from the defective gene function, to hypercalciuria,

TABLE 43.1 Animal Models of Monogenic Hypercalciuria

Genes and gene products	Phenotypes
CLC5[69–72] Chloride channel	• Hypercalciuria • Hyperphosphaturia • Proteinuria • Increased gut Ca absorption • Spinal deformities
NPT2[73] Renal specific Na-coupled phosphate cotransporter	• Hypercalciuria • Hyperphosphaturia • Renal calcifications • Retarded secondary ossification
NHERF-1[74] Na/H exchanger regulatory factor. Docking protein	• Hypercalciuria • Hyperphosphaturia • Hypermagnesuria • Female: reduced BMD and content • Fracture
TRPV5[75] Epithelial calcium channel	• Hypercalciuria • Hyperphosphaturia • Increased intestinal calcium absorption • Reduced trabecular and cortical thickness of bones
VDR[76–78] Vitamin D receptor	• Hypercalciuria on high calcium and lactose diet • Rickets
CalB[78–81] Calbindin-D28k Intracellular calcium buffer	• Normocalcemia • Hypercalciuria • Normocalciuria
NKCC2[82] Na–K–Cl cotransporter	• Hypercalciuria • Polyuria, hydronephrosis • Proteinuria
CAV1[83] Caveolin-1 Scaffolding protein	• Hypercalciuria in male • Bladder stone
AKR1B1[84] Aldo keto reductase	• Hypercalciuria • Hypercalcemia • Hypermagnesemia

BMD, Bone mineral density.

and then to kidney stones. This provides a single point of origin and a single phenotypic endpoint allowing the investigator to utilize them as focal anchors to fill the void in data in between. This sharply contrasts with the scenario where the study of hypercalciuria with reductionistic approaches diverges into manifold pathophysiologic pathways leading to contradiction and confusion.

Note that the pathophysiologic mechanisms of hypercalciuria and/or kidney stones in these conditions are extremely diverse. This broad range of origin of hypercalciuria is also mirrored by the monogenic disorders in both animals and humans. Some of the animal gene deletion experiments were targeting proteins in mineral metabolism and therefore hypercalciuria was expected whereas others encountered hypercalciuria serendipitously (Table 43.1). The human hypercalciuric syndromes are categorized approximately into the origin of the hypercalciuria within the kidney in different nephron segments, as well as extrarenal loci (Table 43.2). This underscores the point made earlier on how defects in many organs can all converge on hypercalciuria as a phenotype.

One perennial question is whether some of these loci have alleles that contribute to the risk in the general hypercalciuric stone-forming population. It is possible that a *forme fruste* of these mutant proteins are polymorphic alleles that are more prevalent in the generation population and affect their functions only slightly but yet impacts on uCa excretion. Multiple loci can then each contribute to small but yet additive effect on calciuria. This monogenic database will be most valuable in tackling the polygenic complex trait of calciuria.

5 ANIMAL MODELS

5.1 A Rodent Model of Polygenic Hypercalciuria

Animal models of stone formation generally are monogenic disorders, as described in Table 43.1, caused by a defect that most often interferes with renal tubular calcium reabsorption, resulting in hypercalciuria and nephrolithiasis. However, human nephrolithiasis clearly is a polygenetic disorder. The majority of human kidney stone formers with Ca-containing kidney stones are hypercalciuric when compared to nonstone formers.[1,3,5,6,85,86] To help understand the mechanism of idiopathic hypercalciuria in man, we developed an animal model of this disorder.[87–118] Through >95 generations of successively inbreeding the most hypercalciuric progeny of the most hypercalciuric Sprague-Dawley (SD) rats, we established a strain of rats that now consistently excrete ~10 times as much uCa as SD controls on all diets and feeding regimens tested (Table 43.3, Fig. 43.1), universally form kidney stones and are termed genetic hypercalciuric stone-forming (GHS) rats.[87–89,91,94,96,97,99,108,110,113]

Compared to SD, GHS rats have a normal serum(s) Ca and absorb far more dietary Ca,[102,108] similar to observations in many humans with IH.[4,5] The increase in intestinal Ca absorption is due to a significant increase in the mucosal to serosal (absorptive) Ca flux with no change in the serosal to mucosal (secretory) flux.[104] When GHS rats are fed a diet essentially devoid of Ca, urine (u) Ca excretion remains significantly elevated compared with that of similarly fed SD, indicating a defect in renal tubule Ca reabsorption or an increase in bone resorption, or both,[117] again similar to observations in humans.[157,198] Cultured bone from GHS rats released more Ca than the bone of SD rats when exposed to increasing amounts of calcitriol.[98] The GHS rats, fed an ample Ca diet, have reduced BMD (Fig. 43.2) with reduced trabecular volume,

TABLE 43.2 Human Monogenic Causes of Hypercalciuria

Diseases	Genes and gene products (gene MIM)	Phenotypes
Predominantly proximal tubule		
Dent's Disease (X-linked recessive nephrolithiasis, X-linked hypophosphatemic rickets, low-molecular weight proteinuria)	*CLC5* Proton/chloride exchanger (300008)	• Hypercalciuria • Hyperphosphaturia • Tubular proteinuria • Nephrocalcinosis • Nephrolithiasis • Rickets
Lowe syndrome (oculocerebrorenal syndrome)	*OCRL* Inositol polyphosphate 5 phosphatase (300535)	• Hypercalciuria • Phosphaturia • Aminoaciduria • Nephrocalcinosis • Nephrolithiasis • Vitamin D resistant rickets
Wilson's disease (hepatolenticular degeneration)	*ATP7B* Copper-transporting ATPase (606882)	• Fanconi syndrome • Hypercalciuria • Hyperphosphaturia • ReTA • Nephrolithiasis • Osteoporosis
Tyrosinemia type 1 (hepatorenal tyrosemia)	*FAH* Fumarylaceto-acetase (613871)	• Fanconi syndrome • Hypercalciuria • Nephrocalcinosis • Nephrolithiasis • Rickets
Glycogen storage disease Type 1a (von Gierke disease, hepatorenal glycogenosis, glucose-6-phosphatase deficiency)	*G6PC* Glucose-6-phosphatase (613742)	• Fanconi syndrome • Hypercalciuria • Nephrocalcinosis • Nephrolithiasis • Osteopenic fracture
Hypophosphatemia nephrolithiasis	*SLC34A1/NPT2A* Sodium–phosphate cotransporter IIa (182309)	• Phosphaturia • Hypercalciuria • Nephrolithiasis • Calcium hyperabsorption • Osteoporosis
Hereditary hypophosphatemic rickets with hypercalciuria	*SLC34A3/NPT2C* Sodium phosphate cotransporter IIc (241530)	• Phosphate wasting • Hypercalciuria • Calcium hyperabsorption • Rickets/osteomalacia
β-thalassemia	*HBB* β-globin (141900)	• Proteinuria • Hypercalciuria • Osteomalacia
Glucose/galactose malabsorption	*SLC5A1/SGLT1* Sodium–glucose cotransporter (182380)	• Glycosuria • Hypercalciuria • Nephrolithiasis • Nephrocalcinosis
Predominantly thick ascending limb		
Familial hypomagnesemia with hypercalciuria and nephrocalcinosis (isolated renal hypomagnesemia)	*CLDN16* Claudin 16 (603959) *CLDN19* Claudin 19 (610036)	• Renal magnesium wasting • Hypercalciuria • Nephrocalcinosis • Heterozygotes more prone to nephrolithiasis
Bartter syndrome		
Type 1 (Antenatal hypokalemic alkalosis with hypercalciuria)	*NKCC2/SLC12A1* sodium–potassium–chloride cotransporter (600839)	• Severe neonatal involvement • Metabolic alkalosis • Hypercalciuria • Nephrocalcinosis

TABLE 43.2 Human Monogenic Causes of Hypercalciuria (*cont.*)

Diseases	Genes and gene products (gene MIM)	Phenotypes
Type 2 (Antenatal hypokalemic alkalosis with hypercalciuria, prostaglandin syndrome)	*KCNJ1/ROMK* Renal outermedullary potassium channel (600359)	• Severe neonatal involvement • Metabolic alkalosis • Hypercalciuria • Nephrocalcinosis • Osteopenia
Type 3 (Classic Bartter's)	*CLCNKB* CLC-KB Kidney chloride channel (602023)	• Mild or no hypercalciuria • No nephrocalcinosis
Type 4A (Sensorineural deafness)	*BSND* Barttin, subunit of chloride channel (606412)	• Congenital deafness • Metabolic alkalosis • Mild or no hypercalciuria • Nephrocalcinosis • Osteopenia
Type 4B (Digenic Bartter's with sensorineural deafness)	*CLCNKA and CLCNKB* CLC-KA and KB Kidney chloride channels (602024) (602023)	• Congenital deafness • Metabolic alkalosis • Mild or no hypercalciuria • Nephrocalcinosis • Osteopenia
Type 5 (Autosomal dominant, hypocalcemia with Bartter syndrome)	*CaSR* Calcium-sensing receptor activating mutations (601199)	• Overlap between autosomal dominant hypocalcemia and Bartter syndrome • Hypocalcemia • Hypercalciuria
Autosomal dominant hypocalcemia	*CaSR* Calcium-sensing receptor activating mutations (601199)	• Overlap between autosomal dominant hypocalcemia and Bartter syndrome • Hypocalcemia • Hypercalciuria
Predominantly distal convoluted and collecting tubule		
Pseudohypoaldosteronism type 2 (Familial hypertensive hyperkalemia)	*WNK4* Lysine-deficient protein kinase 4 (601844)	• Hypercalciuria • Mild metabolic acidosis
RTA		
Distal autosomal recessive RTA with sensorineural deafness	*ATP6V1B1* V-ATPase, B1 subunit of V1 domain (192132)	• Hypercalciuria • Nephrocalcinosis • Nephrolithiasis • Gut hyperabsorption • Bone resorption
Distal autosomal recessive RTA	*SLC4A1* AE1 Anion exchanger; B and 3 (109270)	• Hypercalciuria • Nephrocalcinosis • Nephrolithiasis • May or may not be associated with erythrocyte abnormalities
Distal autosomal recessive RTA with preserved hearing	*ATP6V0A4* V-ATPase, a4 subunit of Vo domain (605239)	• Hypercalciuria • Nephrocalcinosis • Nephrolithiasis • Osteomalacia
Distal autosomal dominant RTA with sensorinerual deafness	*SLC4A1* AE1, Anion exchanger (109270)	• Hypercalciuria • Nephrocalcinosis • Nephrolithiasis
Autosomal recessive Osteopetrosis with RTA (mixed proximal and distal RTA)	*CAII* Carbonic anhydrase II (611492)	• Nephrocalcinosis, • Nephrolithiasis • Osteopetrosis • Brain calcifications
Liddle syndrome	*SCNN1B SCNN1G* Epithelial sodium channel, β (600760) or γ subunits (600761)	• Hypercalciuria • Nephrocalcinosis

(*Continued*)

TABLE 43.2 Human Monogenic Causes of Hypercalciuria (*cont.*)

Diseases	Genes and gene products (gene MIM)	Phenotypes
Extrarenal		
Congenital sucrase-isomaltase deficiency	*SI* Sucrase-isomaltase (609845)	• Calcium hyperabsorption • Hypercalcemia • Hypercalciuria • Nephrocalcinosis
Congenital lactase deficiency (congenital alactasisa, disaccharide intolerance)	*LCT* Lactase (603202)	• Calcium hyperabsorption • Hypercalcemia • Hypercalciuria • Nephrocalcinosis
Multiple endocrine neoplasia type I with hyperparathyroidism	MEN1 Menin (613733)	• Hyperparathyroidism • Increased bone turnover • Hypercalciuria • Nephrocalcinosis • Nephrolithiasis
Metaphyseal chondrodysplasia Murk Jansen type (metaphyseal dysostosis)	*PTHR1* Parathyroid hormone receptor-1 (168468)	• High bone resorption with metaphyseal deformitites • Hypercalcemia • Hypercalciuria • Hyperphosphaturia
McCune–Albright syndrome (polyostotic fibrous dysplasia)	*GNAS1* Guanine nucleotide binding protein (G protein) α-1 (139320)	• Hypercalciuria • Hyperphosphaturia • Nephrocalcinosis • Fibrous dysplasia, rickets, craniofacial hyperostosis
Infantile hypophosphatasia (phosphoethanolaminuria)	*ALPL* Nontissue-specific alkaline phosphatase (171760)	• Hypercalcemia • Hypercalciuria • Nephrocalcinosis • Rickets, craniosynostosis
Cystic fibrosis	*CFTR* Cystic fibrosis transmembrane conductance regulator (602421)	• Hypercalciuria • Hypocitraturia • Hyperoxaluria • Nephrocalcinosis
Osteogenesis imperfecta Type 1	*COL1A1,COL1A2* Collagen type 1, α1 and 2 (120150, 120160)	• Fractures • Hypercalciuria
Beckwith–Wiedemann syndrome (exomphalos–macroglossia–gigantism syndrome)	*CDKN1C* Cyclin-dependent kinase inhibitor 1C, p57(KIP2) (600856) *NSD1* Nuclear receptor binding SET domain protein 1 (606681) *H19* H19 gene, Adult skeletal muscle gene-1 (103280) *KCNQ10T* Long QT intronic transcript- 1(604115)	• Hypercalciuria • Nephrocalcinosis • Nephrolithiasis
Unknown loci or polygenic complex		
Williams–Beuren syndrome *(not monogenic)*	7q11.23 deletion: *ELN*/elastin *LIMK1*/LIM kinase 1 *RFC2*/replication factor C, subunit 2	• Infantile hypercalcemia • Hypercalciuria • Rickets
Hyperabsorptive hypercalciuria	4q33-qter deletion	• Calcium hyperabsorption • Skeletal abnormalities • Hypercalciuria • Nephrocalcinosis

TABLE 43.2 Human Monogenic Causes of Hypercalciuria (*cont.*)

Diseases	Genes and gene products (gene MIM)	Phenotypes
Down's syndrome	Trisomy 21	• Calcium hyperabsorption • Low mineral bone density Hypercalciuria
Blue diaper syndrome (familial hypercalcemia with nephrocalcinosis and indicanuria)	Autosomal recessive or X-linked. Possibly *SLC16A10* T-type amino acid transporter	• Hypercalcemia • Hypercalciuria • Nephrocalcinosis • Indicanuria
Neonatal selflimited primary hyperparathyroidism	Gene unknown. Autosomal recessive (?)	• Increased bone turnover • Hypercalciuria • Nephrocalcinosis • RTA
Bilateral macular coloboma with hypercalciuria	Gene unknown Autosomal recessive	• Hypercalciuria

Phenotype: Only features related to hypercalciuria is shown. The full syndromes have more complex phenotypic features. RTA, Renal tubular acidoses.

TABLE 43.3 Comparison of Mineral Metabolism Related Characteristics of Hypercalciuric Stone-Forming Humans With Genetic Hypercalciuric Stone Forming Rats

	Hypercalciuric stone-forming (IH) humans	GHS rats
Urine Ca excretion	Increased (by definition)	Increased[85,87–105,114,116–118,120]
Intestinal Ca absorption	Increased in most patients[4,5,121–144]	Increased[87–105,117]
Renal tubular Ca reabsorption	Decreased in many patients[145–147]	Decreased[95]
Bone resorption	Increased in most patients—as evidenced by markers of bone resorption[148–154]	Increased[94,98,113–115,155,156]
Bone mineral density	Decreased in most patients[4,121,154,157–165]	Decreased[114,118]
Fracture propensity	Increased[166,167]	Increased[118]
Serum Ca	Normal[4,5]	Normal[102,108,113,114]
Serum PTH	Normal to reduced[132,163,168–170] or elevated[168,169]	Reduced[120]
Serum $1,25(OH)_2D_3$	Normal to elevated [126,131,132,163,171–175]	Normal to elevated[93,101,106,117,176]
VDR	Increased number[177] or no increase[45] Gene polymorphisms [37,38,40,42,43,46,178–185]	Increased number[93,98,101,176,186]
Biological activity of VDR	Unknown	Increased activity[113–115]
Ca Receptor	Changes in number not reported Activating and inactivating mutations associated with hyper- and hypocalciuria, respectively.[16,187] Gene polymorphisms[48,188]	Increased number[106] Treatment with cinacalcet activates the receptor—associated with increased uCa in SD but not GHS rats[120]
Stone formation	Consequence of hypercalciuria [4,5,122–124,126,189–196]	Universal[87–89,94,96,97,99,197]

PTH, parathyroid hormone; SD, Sprague Dawley; uCa, urinary calcium; VDR, vitamin D receptor.

mineralized volume, and thickness, and their bones are more brittle and fracture prone, indicating that GHS rats have an underlying bone defect.[118]

Administration of a bisphosphonate, which inhibits bone resorption, to hypercalciuric rats fed a low-Ca diet (LCD) significantly reduces urinary Ca excretion.[94] Utilizing clearance studies, a primary defect in renal Ca reabsorption is observed.[95] Thus, these hypercalciuric rats have a systemic abnormality in Ca homeostasis; they absorb more intestinal Ca, they resorb more bone, and they do not adequately reabsorb filtered Ca (Fig. 43.3).

We have found that the bone, kidney, and intestine of our inbred strain of hypercalciuric rats have an increased number of vitamin D receptors (VDR) and CaSR.[98,101,106,109,176] We demonstrated an exaggerated response of VDR gene expression to $1,25(OH)_2D_3$

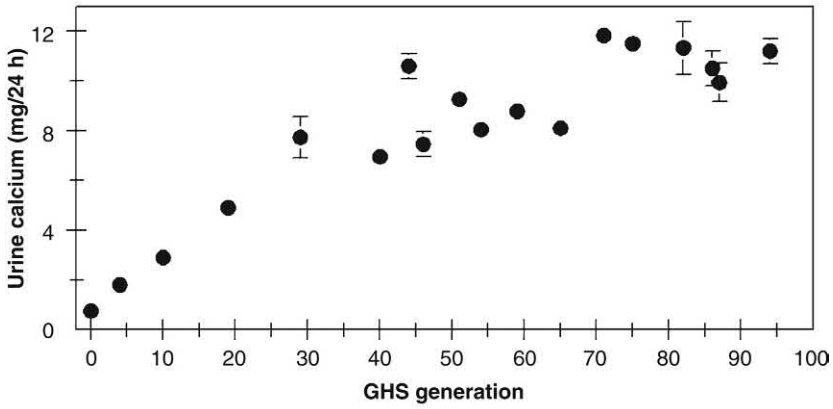

FIGURE 43.1 Through successive inbreeding of the most hypercalciuric progeny of SD rats we have established a strain of genetic hypercalciuric stone-forming (GHS) rats that consistently excrete ~ 8–10 times as much urinary Ca as the parental strain. All data from published studies.[87–112,117–119]

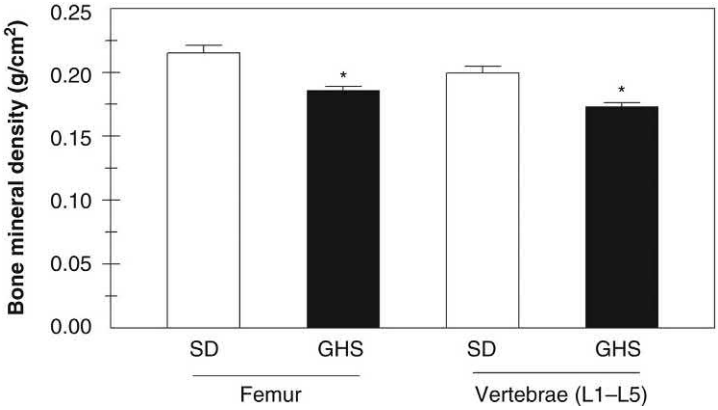

FIGURE 43.2 GHS rats have a reduction in BMD in femur and vertebrae even when fed a normal Ca diet.[42]

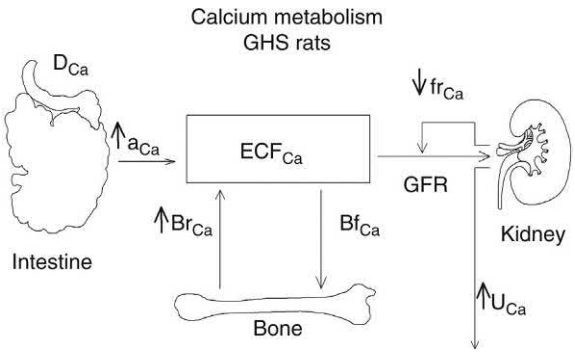

FIGURE 43.3 Systemic abnormality in Ca homeostasis in GHS rats: increased intestinal Ca absorption, increased bone resorption, and decreased Ca reabsorption.[116]

in intestine and kidney in GHS rats.[93] The injection of a single dose of $1,25(OH)_2D_3$ increased transcription of VDR, CaSR, and TRPV6.[106,199–202] $1,25(OH)_2D_3$ stimulated CYP24A1 (25-OH-24-OHase)[203,204] equally in GHS and SD rat kidneys and CYP27B1 (25-OH-1α-OHase) transcription was suppressed in GHS versus SD rats, respectively.[205] The results suggest that a greater number of VDR–$1,25(OH)_2D_3$ complexes bind to multiple regions in the promoter regions of the above VDR-dependent genes in GHS rats to stimulate or repress gene expression. We have found that the elevated levels of VDR are regulated by a decreased level of the transcription factor Snail,[111] suggesting potential underlying mechanism(s) for the hypercalciuria. In a clinical study, circulating monocytes from humans with idiopathic hypercalciuria were shown to have an increased number of VDR[177]; however, we do not know if hypercalciuric humans have altered levels of Snail.

As GHS have normal levels of $1,25(OH)_2D_3$ (1,25D), the higher levels of VDR suggest that their VDR is undersaturated with 1,25D. We tested the hypothesis that 1,25D would induce a greater increase in uCa in GHS.[113] After injecting 1,25D, uCa in SD and GHS increased; however the increased uCa with 1,25D in GHS exceeded that of SD, indicating that the increased VDR in GHS induces a greater biological response. This increase in uCa must come from the intestine and/or bone.

To determine whether the excess uCa was derived primarily from diet or from increased bone resorption, we asked whether 1,25D would increase uCa in GHS fed a LCD.[155] On LCD with 1,25D, uCa in SD increased; however, uCa again increased significantly more in GHS. In GHS rats on LCD with or without 1,25D, uCa far exceeded daily Ca intake implying a loss of bone mineral.

To determine the role of bone resorption in the increased uCa in GHS rats, we tested the hypothesis that LCD, coupled to inhibition of bone resorption by the bisphosphonate alendronate (alen), would eliminate the enhanced 1,25D-induced hypercalciuria in GHS.[115] Alen eliminated the 1,25D-induced increase in uCa in SD. However, in GHS alen decreased, but did not eliminate, the 1,25D-induced hypercalciuria, suggesting maximal alen cannot completely prevent the 1,25D-induced bone resorption in GHS. These results confirm the role of increased bone resorption in the hypercalciuria of GHS rats.

To study the effect of the increased VDR on the osseous response to 1,25D, we fed GHS and SD rats an ample Ca diet and injected either low or high dose 1,25D or vehicle daily.[114] With 1,25D there was a mineralization defect and a loss of BMD in GHS rats that exceeded changes in SD and contributed to increased hypercalciuria, suggesting that these bones would be more fracture prone. The enhanced effect of 1,25D in GHS rats indicates that the increased VDRs are biologically active and supports our hypothesis that in GHS at baseline, VDR remains available for stimulation by exogenous 1,25D.

Regions of five chromosomes, 1, 4, 7, 10, and 14, were found to be associated with the hypercalciuria[92] (Fig. 43.4).

The specific genes responsible for the hypercalciuria have not yet been identified. Normocalciuric Wistar–Kyoto rats were then bred with the GHS rats to yield congenic rats with the chromosome 1 locus on the Wistar–Kyoto background.[109] These congenic rats were also hypercalciuic; but to a lesser extent than the parenteail GHS rats supporting the importance of this locus and that the hypercalciuria in the GHS rats is due to a number of genes (Fig. 43.5).

5.2 Stone Formation in Genetic Hypercalciuric Rats

After eating standard rat chow (1.2% Ca) for 18 weeks virtually all of these hypercalciuric rats form kidney stones while there is no evidence for stone formation in SD.[99] The stones contain only calcium and phosphate, without oxalate, and by X-ray diffraction the stones are exclusively poorly crystalline apatite which is composed of calcium and oxalate.[88,89,96,99,197] When fed additional hydroxyproline, an amino acid which is metabolized to oxalate,[206] these rats formed calcium oxalate kidney stones,[87,91] the most common kidney stones formed by man.[87,91] As each of the hypercalciuric rats forms renal stones, they have been termed GHS rats.[87–89,91,94,96,97,99,108] The pathophysiology responsible for the hypercalciuria parallels that found in hypercalciuric humans and is thus an excellent model of hypercalciuria (Table 43.3).

5.3 Decreased Bone Density in Hypercalciuria

5.3.1 *Humans*

Patients with idiopathic hypercalciuria often excrete more calcium than they absorb indicating a net loss of total body calcium.[4,5,121–124,189,207,208] The source of this additional urine calcium is almost certainly the skeleton, the largest repository of body calcium.[157,171,209] Idiopathic hypercalciuria has been associated with markers of increased bone turnover.[148–150] Urinary hydroxyproline is increased in unselected patients with idiopathic hypercalciuria,[148] and serum osteocalcin levels are elevated in stone formers who have a defect in renal tubule calcium reabsorption but not in those with excessive intestinal calcium absorption.[149] Studies with[47]calcium demonstrate increased bone formation and resorption with the latter predominating.[125] Cytokines known to increase bone resorption have also been shown to be elevated in patients with idiopathic hypercalciuria.[151–154]

BMD is correlated inversely with urine calcium excretion in both men[210] and women.[211] This relationship was confirmed in stone formers but not in nonstone formers.[209] A number of studies demonstrate that patients with nephrolithiasis have a reduction in BMD compared to matched controls.[4,121,150,154,157–163,166,212] After adjusting for a large number of variables, an analysis of the third National Health and Nutrition Examination SurveyNHANES III demonstrated that men with a history of kidney stones have a lower femoral neck BMD than those without a history of stones.[167] Analysis of almost 6000 older men again demonstrated an association of kidney stones with decreased BMD.[213] In addition to lower mineral density, stone formers actually have an increased risk of fractures.[166,167] In National Health and Nutrition Examination Survey there was an increased risk of wrist and spine fractures in stone formers[167] and, in a retrospective analysis, stone formers had an increased incidence of vertebral fractures, but not fractures at other sites.[166]

5.3.2 *Hypercalciuric Rats*

We have recently demonstrated that GHS rats have a reduction in BMD and bone strength even when fed a diet with ample calcium[118] (Table 43.4, Fig. 43.2).

GHS rats had reduced cortical (humerus) and trabecular (L(1)–L(5) vertebrae) BMD and had a decrease in trabecular volume and thickness. GHS rats had no change

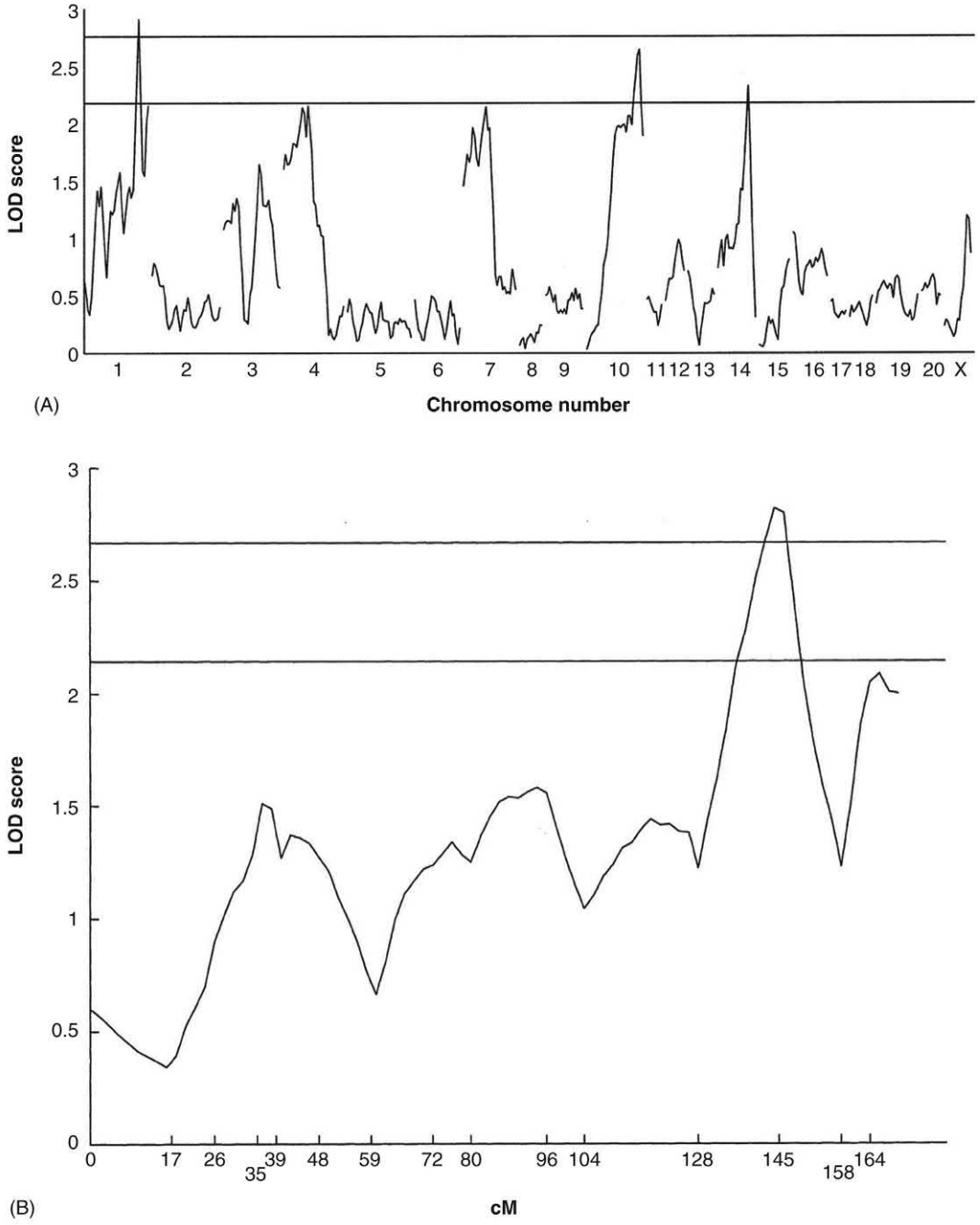

FIGURE 43.4 (A) One-dimensional genome scan with gender and cross as additive covariates. (B) One-dimensional scan of chromosome 1. On both panels, horizontal lines indicate thresholds for significance (lower line "suggestive," upper line "significant") for the LOD score based on 1000 permutations.[92]

in vertebral strength (failure stress), ductibility (failure strain), stiffness (modulus), or toughness, whereas in the humerus, there was reduced ductibility and toughness and an increase in modulus, indicating that the defect in mechanical properties is mainly manifested in cortical, rather than trabecular bone. In the GHS rat, the cortical bone is more mineralized than the trabecular bone. Thus, the GHS rats, fed an ample calcium diet, have reduced BMD with reduced trabecular volume, mineralized volume, and thickness, and their bones are more brittle and fracture prone, indicating that GHS rats have an intrinsic disorder of bone not related to a deficiency in dietary calcium.

Thiazide diuretic agents, such as chlorthalidone (CTD) reduce urine calcium excretion in normals,[214] patients with hypercalciuria,[215] and rats.[107,216] These drugs

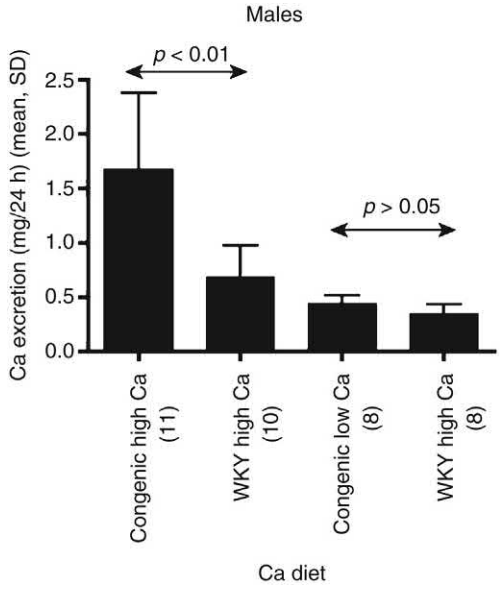

FIGURE 43.5 Calcium excretion phenotype for male congenic and control WKY rats on high- and low-calcium diets (LCD). Each bar represents the mean excretions values for four 24 h measurements for rats on the high or the LCD. Numbers of rats in each group are indicated in parentheses.[109]

act by stimulating calcium reabsorption in the distal convoluted tubule[214] and by producing extracellular fluid volume depletion.[217] Thiazide diuretics are used to treat calcium oxalate stone disease[1,4]; a metaanalysis revealed that in studies of more than 2 years duration, there was a significant reduction in stone recurrence rate.[218] A number of studies have shown that when thiazides are used to treat hypertension[219] there is a reduction of osteoporotic fractures[220,221] and often an increase in BMD.[222,223]

We used GHS rats to test the hypothesis that CTD would have a favorable effect on BMD and bone quality. GHS rats were fed an ample calcium diet and half were also fed CTD.[112] As expected CTD reduced uCa in GHS rats.[107,216,224] In the axial and appendicular skeletons an increase in trabecular mineralization was observed with CTD compared to controls.[112] CTD also improved the architecture of trabecular bone. Using μCT, trabecular bone volume, trabecular thickness and number were increased with CTD. A significant increase in trabecular thickness with CTD was confirmed by static histomorphometry. CTD also improved the connectivity of trabecular bone. Significant improvements in vertebral strength and stiffness were measured by vertebral compression. Conversely, a slight loss-of-bending strength was detected in the femoral diaphysis with CTD. These results obtained in hypercalciuric rats suggest that CTD can favorably influence vertebral fracture risk.

The primary endpoint for successful treatment of patients with calcium-containing kidney stones is a decrease in the rate of stone recurrence.[1,5,85,86] While decreasing stone formation is an important goal, what should concern clinicians equally is maintaining and improving the patient's BMD and bone quality.[212] As stated earlier, stone formers have both a reduction in BMD[4,121,150,154,157–163,166,167,212] and an increase in fracture rate compared to nonstone formers.[166,167] While acute stone episodes often are resolved quickly, patients may live the remainder of their lives in pain and with reduced function due to the osseous complications related to fractures and the risk of further fractures.[225]

6 CLINICAL PATHOPHYSIOLOGY

As indicated earlier, the definition of hypercalciuria is generally considered as >250 mg (6.25 mmoL)/24 h in males or >200 mg (5 mmoL)/24 h in females or >4 mg (0.1 mmoL)/kg/24 h for both gender for adults and 4 mg/(0.1 mmoL) kg/day, or a uCa/creatinine ratio higher than 0.2 for children.[9] However, urine calcium excretion is a continuous variable and the lithogenic potential of a given amount of uCa is important only in relation

TABLE 43.4 Bone Parameters on GHS Rats

	SD	GHS rat NCD	GHS rat LCD	GHS rat NCD + CTD
In vivo:				
Bone strength	Nl	Decreased[118]	Decreased versus NCD Decreased versus SD on LCD[118]	Increased versus GHS NCD[112]
BMD	Nl	Decreased[118]	Decreased versus NCD[118]	No change versus GHS[112]
Bone volume	Nl	Decreased[118]	Decreased versus SD on LCD[118]	
Fracture risk	Nl	Increased[118]	Increased[118]	
In vitro:				
$1,25(OH)_2D_3$ stimulation of bone Ca efflux	Nl	Increased[98,113,115]		
PTH stimulation of bone Ca efflux	Nl	Nl[98]		

BMD, Bone mineral density; CTD, chlorothalidone; LCD, low calcium diet (0.02% calcium); NCD, normal calcium diet (1.2% calcium); Nl, normal.

to its liquid environment. The amount of volume that is diluting the calcium, the amount of citrate that is binding the calcium in a soluble complex, the urinary pH that helps to determine the driving force for forming a solid phase, and the other ions that are excreted, such as oxalate and phosphate, are all important determinants of the lithogenic potential of a specific amount of uCa.

Pak et al. devised a research protocol to distinguish between reduced renal tubule calcium reabsorption (renal hypercalciuria) and increased intestinal calcium absorption (absorptive hypercalciuria).[145–147] Patients are put on a low calcium diet (LCD) (400 mg calcium and 100 mEq NaCl/day) for 7 days, and a 24 h urine is collected. Fasting urine calcium excretion is measured and again after a 1 g oral calcium load. Renal hypercalciuria is defined as an elevated fasting urine calcium (>0.11 mg/100 mL glomerular filtration) and an elevated PTH. Absorptive hypercalciuria is empirically divided into two groups: Type I has normal fasting urine calcium, elevated urine calcium after the oral calcium load, normal PTH, and hypercalciuria on the calcium-restricted diet. Type II is similar except for normalization of uCa on the calcium-restricted diet. In 241 consecutive stone-forming patients they found 24% with Type I absorptive hypercalciuria, 30% with Type II absorptive hypercalciuria, and 8% with renal hypercalciuria.[147] This categorization may reflect different underlying pathophysiology but the distinction is not always clear cut and this type of evaluation is only feasible in a research setting.

To determine the mechanism of hypercalciuria, Coe et al[157] studied 24 patients with idiopathic hypercalciuria and 9 control subjects fed a LCD (2 mg/kg/day) for a week. There was no difference in serum calcium or calcitriol levels; however, the hypercalciuric patients had a mild decrease in PTH levels. On this LCD the controls excreted less calcium then they ingested while 16 of the 24 hypercalciuric patients excreted more calcium than they consumed indicating probable bone loss. There was no clear demarcation between those who retained calcium (surrogate of enhanced intestinal calcium absorption), and those who lost calcium (surrogate of failure of the kidney to reabsorb calcium). In this study, the continuum in urine calcium excretion and in net calcium retention suggested that there were not specific, well-demarcated pathophysiologic etiologies of hypercalciuria in these patients. As patients may not be able to reproducibly classified, even in a clinical research setting, the prescription of a diet low in calcium to those who you believe absorb excessive amounts of dietary calcium can potentially lead to a dangerous reduction of BMD especially in women.[157,209,226] Furthermore, a diet low in calcium promotes, rather than retards, stone formation.[227]

Adams et al.[228] demonstrated that administering calcitriol to normal men on a normal or high calcium diet lead to an increase in both intestinal calcium absorption and urine excretion, in the absence of hypercalcemia, paralleling observations in patients with idiopathic hypercalciuria. When normal men were fed an extremely LCD, administration of calcitriol also led to an increase in intestinal calcium absorption and urine calcium excretion but also to a marked decrease in calcium retention[229] which can only result from enhanced bone resorption. These studies indicate that critical physiologic aspects of idiopathic hypercalciuria can be modeled by calcitriol administration suggesting that an excess of this hormone, or its activity, may be responsible, at least in part, for this clinical disorder. Pak et al. evaluated 300 non-stone-forming patients, 208 patients with absorptive hypercalciuria type I (high intestinal calcium absorption), and 234 stone formers without absorptive hypercalciuria on a constant restricted diet, and showed a clear demarcation between uCa excretion of kidney stone formers with absorptive hypercalciuria type I and normal individuals. Thus, when dietary variables are controlled, the classic definition of hypercalciuria of nephrolithiasis appears valid.[230]

In the genetic hypercalciuric rats, whose physiology closely parallels that of patients with idiopathic hypercalciuria, there is an excess number of receptors for calcitriol.[93,98,101,106,108,176] Favus et al. found increased number of vitamin D receptors in peripheral blood monocytes from male patients with idiopathic hypercalciuria, in the absence of elevated levels of calcitriol, compared to age-matched controls.[177] Evidence points to a systemic dysregulation of calcium transport, rather than a specific organ centered defect, in most patients with idiopathic hypercalciuria. That a small excess of calcitriol can mimic many aspects of this disorder suggests that slight excesses of this hormone, or its receptor, may be integral in the pathophysiology of idiopathic hypercalciuria.

7 COUNSELING AND TREATMENT

With the exception of the rare Mendelian monogenic forms of hypercalciuria or kidney stones, there is currently no formal genetic counseling offered to patients with complex polygenic forms of this disease. A family history should always be inquired but the polygenic nature and multiple confounding nongenetic variables render the construction of a pedigree with genuine genotype–phenotype correlation rather difficult. Whenever possible, hypercalciuria will be preferred over stones as a phenotype. In kindreds with clear familial hypercalciuria and calcium stones, judicious screening and testing of unaffected individuals are justified and should be decided on individual basis. The same applies to genetic testing of candidate loci which should be individualized.

The principle of managing hypercalciuria and kidney stones is akin to that of primary hypertension. Although

in the vast majority of the instances, we do not know the cause of, nor can we cure primary hypertension, we can control the blood pressure and ameliorate the cardiovascular, cerebral, and renal consequences. The treatment of hypercalciuria is also empirical in nature; lower the uCa and thus the risk for stone formation. Currently, the only medical therapy that effectively reduces uCa is thiazide diuretics.[7] The mechanism of how thiazide reduces uCa is multifold which involves more than mere extracellular volume contraction.[231]

The effects of thiazides on calciuria and the hard clinical outcome of recurrent calcium stone formation were tested in randomized control trials.[232–237] In four of these studies (total of ~460 patient-years), thiazide decreased stone recurrence[232,234–236] in populations that were heterogeneous. In three studies, hypercalciuria was reported in 20%–100% of the subjects.[232,234–236] In contrast, two randomized control trials conducted with 75 patient-years concluded that thiazide is ineffective in reducing kidney stone incidence.[233,237] This may in part be due to small sample size, short study duration, and lack of control for fluid and dietary intake. The randomized controlled trials were consistent with open studies totaling over 6600 patient-years of thiazide treatment for calcium nephrolithiasis.[190,191,238–242] The incidence of side effects on thiazide diuretics is approximately 25%–30% although side effects requiring discontinuation are extremely infrequent.[23] Whether long-term thiazide has the same undesirable side effects in stone formers as in hypertensive patients remains to be examined. The thiazide used includes hydrochlorothiazide (50 mg/day), CTD (25–50 mg/day), or indapamide (1.2–2.5 mg/day).

In a short-term trial, bisphosphonates were shown to lower uCa excretion.[34,35] However, no long-term randomized controlled trials have been performed to test the efficacy of these agents. Oral sodium cellulose phosphate was used to inhibit intestinal calcium absorption but it also unfavorably alters urinary magnesium and oxalate, and works only in hyperabsorptive patients, which offsets its efficacy to selected patients where complicated and vigilantly monitored protocols are feasible.[243] Sodium thiosulphate was shown to reduce stone frequency in an uncontrolled human trial and calcium phosphate stone formation in a rodent polygenic model.[110,244,245]

Besides pharmacologic therapy, dietary management has received spotlighted attention as this mode of therapy is popular in certain selected patients while noncompliance is a serious limiting factor in other patients. The panacea of dietary modification for kidney stones is water intake which has been proven uniformly in metabolic studies,[246] epidemiologic analyses,[247] and intervention trials.[248] This is probably the only mode of lifestyle-based therapy that has been proven to work in all three of these formats. The other major factors are dietary calcium, sodium, and protein or acid. Dietary restriction of calcium is generally not recommended due to the risk of osteoporosis, and the modest effect of dietary calcium on hypercalciuria. At any level of dietary calcium intake, stone patients absorb more calcium but most dietary manipulations are on the rather flat part of the response curve yielding limiting returns.[230] There is epidemiologic data supporting that the relative risk of kidney stones is stable from dietary calcium ranging from >1 g down to 600 mg but when intake falls below 600 mg/day, relative risk increases.[247] Dietary sodium induces physiologic hypercalciuria even in normal individuals and stone formers may have an exaggerated response.[10] In the one seminal dietary intervention trial,[227] Borghi et al. made three simultaneous dietary adjustments of salt restriction, protein restriction, and liberalization of calcium intake. In the control group, a selfvolunteered fall in dietary sodium excretion of 30 mmoL/day led to a 4.8 mmoL/day fall in uCa excretion while the treated group reduced their urinary sodium by ~120 mmoL/day out of the prescribed 200 mmoL/day reduction and lowered their uCa by 5.6 mmoL/day. The effect of high protein intake increasing hypercalciuria is well documented in metabolic studies[249] and epidemiologic data is agreement with that although an unusual biphasic curve was documented on the relationship between stone incidence and estimated protein intake.[247] In the large interventional trial mention above where multiple changes were made simultaneously, the reduction in sulfate excretion by 8 mEq/day was associated with a fall in uCa of 5.6 mmoL/day versus a 4.8 mmoL/day. The effect of protein on hypercalciuria was related to the acid content of the food in rodents[250] but in humans, the hypercalciuric effect may be mediated by nonacid components of the protein.[14]

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SECOND EDITION

GENETICS OF BONE BIOLOGY AND SKELETAL DISEASE

EDITED BY **RAJESH V. THAKKER, MICHAEL P. WHYTE, JOHN A. EISMAN, AND TAKASHI IGARASHI**

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