Advances in Experimental Medicine and Biology 1266

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Stem Cell-based Therapy for Neurodegenerative Diseases

Springer

Advances in Experimental Medicine and Biology

Volume 1266

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2019 Impact Factor: 2.450
5 Year Impact Factor: 2.324

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-981-15-4369-2 ISBN 978-981-15-4370-8 (eBook)
https://doi.org/10.1007/978-981-15-4370-8

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

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Fabin Han and Paul Lu

1 Introduction

Human neurodegenerative diseases (NDs) are a group of diseases or disorders in which the majority of neural cells in the nervous system gradually degenerate or die, such as in Alzheimer's disease (Frere and Slutsky 2018), Parkinson's disease (Cacabelos 2017; Dawson et al. 2018), Huntington's disease (Rüb et al. 2016), amyotrophic lateral sclerosis (Hardiman et al. 2017), and spinal muscular atrophy (Nash et al. 2016). In some neurodegenerative diseases, glial cells, such as oligodendrocytes, in multiple sclerosis are lost (Huang et al. 2017) whereas in retinal degenerative diseases, the retinal progenitor cells or photoreceptor cells are degenerated (MacLaren et al. 2006; Nazari et al. 2015). Unfortunately, the neural cells in the central nervous system (CNS), especially neurons, can hardly be regenerated after degeneration or death, resulting in permanent functional abnormality (Barker and de Beaufort 2013; Fu et al. 2018). With the constant increase in life expectancy, neurodegenerative diseases are a serious problem for the society and our understanding on the mechanisms of these disorders has not been sufficient to provide an effective treatment for the millions of patients with NDs worldwide.

2 Advance in Molecular Pathology of Neurodegenerative Diseases

Millions of people are affected by neurodegenerative diseases worldwide, and the most common types are Alzheimer's disease and Parkinson's disease. It is estimated that about 50 million people developed dementia with Alzheimer's disease and more than ten million people are living with Parkinson's disease worldwide. One clear fact is that the incident of most neurodegenerative diseases increases with age except for spinal muscular atrophy (Barker et al. 2018; Erkkinen et al. 2018). Since the CNS has very limited regeneration capacity, it is an urgent need to develop effective treatments to slow down the progression of neurodegeneration or replace degenerated neurons and glia for the restoration of neural functions in patients with NDs.

The recent advance on the molecular mechanisms of NDs revealed that the aging of

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F. Han, P. Lu (eds.), *Stem Cell-based Therapy for Neurodegenerative Diseases*, Advances in Experimental Medicine and Biology 1266, https://doi.org/10.1007/978-981-15-4370-8_1

brain is the main risk factor for the development of neurodegenerative diseases (NDs) which are characterized by progressive neuronal death and loss of specific neuronal populations. The pathological studies showed that each of NDs usually affects particular neurons to produce selective neuronal loss in some specific regions of the brain. The molecular mechanisms underlying the selective neuronal and regional vulnerability have not been completely understood. However the recent technologies, such as single cell sequencing, whole-genome analysis, and the various animal models that generate spatial and adult onset temporal features of the pathology, help to identify the intrinsic morphological, electrophysiological, and biochemical properties of specific neurons of neurodegenerative diseases (Jansen et al. 2019; Welch et al. 2019; Jaehoon Shin 2015). As it is shown in Fig. 1.1, Parkinson's disease (PD) is mainly affected by the dopamine neuron loss in substantial nigra; Alzheimer's disease (AD) is affected by pyramidal cholinergic neurons in hippocampi; Huntington's disease (HD) is associated with interneuorons; and the motor neurons are mainly affected in amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA).

3 Advance in Stem Cell Replacement Therapies for Neurodegenerative Diseases

In the development of organisms, stem/progenitor cells persist in certain tissues or organs as a repair and replenishing system, such as neural stem cells in specialized niches of the mammalian brain (Silva-Vargas et al. 2013). Newly generated neurons in adult can integrate into a pre-existing neuronal network. A recent live imaging study demonstrated that long-term cell division from an proliferative progenitor for generation of new neurons continues in mouse hippocampus, indicating continuous neurogenesis in hippocampus of brains (Pilz et al. 2018). Therefore stem cell replacement to the diseased or injured brain cells has great therapeutic potential for curing neurodegenerative diseases. Neural stem cells (NSCs) transplanted to the specific host brain regions can also create a favorite microenvironment to protect the surrounding host neural cells from degeneration. More importantly, NSCs or neural progenitor cells (NPCs) are able to differentiate into specific neural cells, including neurons and glia, which could replace degenerated neurons and glial cells and reconstruct the functional neural circuits in NDs (Upadhyay et al. 2015).

The earlier cell transplantation for PD started in the 1980s and showed that transplantation of fetal brain tissue containing the precursor cells of dopamine neurons could relieve the symptoms of PD patients. Hereafter many cell types have been explored for the treatment of PD and other NDs using different stem cells. Since human embryonic stem cells (hESCs) are derived from the inner cell mass of blastocysts, they are able to be induced to any kind of neural stem cells and neurons for the treatment of NDs (Thomson et al. 1998; Martello and Smith 2014). In order to overcome the allogeneic rejection of ESCs and fetal stem cells, recent development of induced pluripotent stem cells (iPSCs) which are reprogramed from adult somatic cells (Takahashi et al. 2007) paved the way of stem cells to the clinical application (Blau and Daley 2019). The iPSCs can also be used for modeling the disease pathology for drug screening and can be transplanted back to the patients themselves as they are derived from autologous somatic cells to reduce the risk of immune rejection of implanted cells (Badja et al. 2014; Han et al. 2015a). Besides ESCs and iPSCs, other multipotent stem cells are also able to self-renew and differentiate into multiple specialized cell types present in a specific tissue or organ. These multipotent stem cells can also be isolated from developing fetal tissues or directly from some adult tissues. During the development of CNS, NSCs enter an intermediate stage called neural progenitor cells (NPCs) that gradually specify as neuronal or glial linage restricted precursor cells, named as neuronal restricted precursors (NRP) or glial restricted precursors (GRP) that are capable of self-renewal and differentiating into neurons and glia, respectively

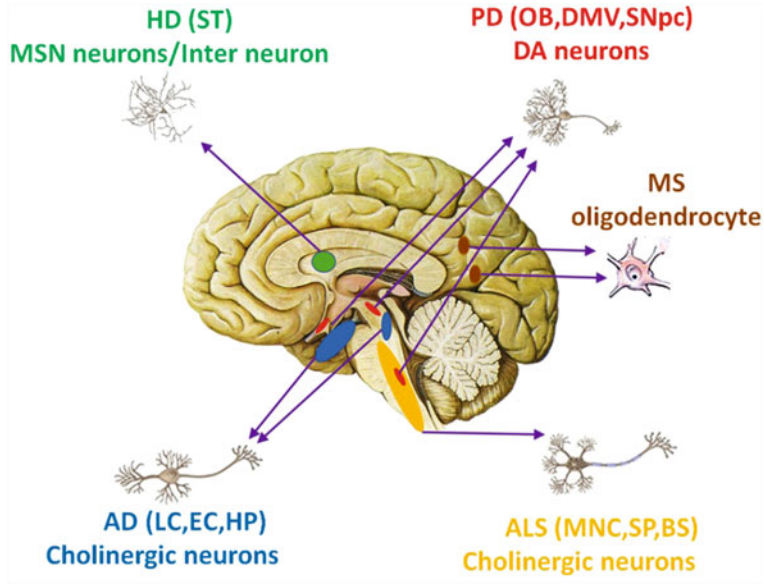

Fig. 1.1 Regions and neurons that are affected in neurodegenerative diseases. The affected regions in different neurodegenerative diseases are shown in different colors. LC, locus coeruleus; HP, hippocampus; OB, olfactory bulb; DMV, dorsal motor nucleus of the vagus; MNC, motor neocortex; SP, spinal cord; BS, brainstem; FI, frontal insula; DG, fascia dentata of the dentate gyrus; ST, striatum. (Reproduced from Fu et al. 2018 with permission)

(Mayer-Proschel et al. 1997; Rao and Mayer-Proschel 1997; Bond et al. 2015). At certain developmental stages or cell culture conditions, NSCs and NPCs may co-exist as a mixture of neural stem cells and neural progenitor cells. NPCs are mostly isolated from developing CNS whereas NSCs are derived from ESCs or iPSCs (Gage and Temple 2013; Tao and Zhang 2016; Han et al. 2015a). These NSC-formed neurospheres in culture can differentiate into all three lineages of neural cells, neurons, astrocytes, and oligodendrocytes, as shown in Fig. 1.2.

Besides neural stem cells, bone marrow-dervied mesenchymal stem cells (BM-MSCs) and umbilical-derived mesenchymal stem cells (UC-MSC) are typical adult multipotent stem cells which can also differentiate to neural cells, muscle cells, and osteocytes (Méndez-Ferrer et al. 2015; Han et al. 2018). BM-MSCs are originally isolated and transplanted for the treatment of hematopoietic cancers (leukemia) and non-hematopoietic malignancies after high-dose chemotherapy. Besides their capacity to generate blood-forming cells, mesenchymal stem cells have been shown to have multilineage differentiation capacity in vitro, including differentiation into osteoblasts, chondrocytes, and adipocytes. BM-MSCs were transplanted for the treatment of neurodegenerative diseases due to their multilineage differentiation capacity, including potential neural cells, modulation of inflammation, neurotrophic factor mediated neuroprotection, enhanced neurogenesis, and abnormal protein aggregate clearance (Volkman and Offen 2017). UC-MSCs are also transplanted for treating the neurological diseases as multipotent stem cells from umbilical cord blood can differentiate into hematopoietic, epithelial, endothelial, and potential neural progenitor cells (Achyut et al. 2014; Batsali et al. 2013; Han et al. 2018). Because of their easy access from multiple resources, UC-MSCs are attractive for tissue engineering and regenerative medicine, including the treatment of neurodegenerative diseases.

In addition to BM-MSCs and UC-MSCs, similar kinds of multipotent stem cells can also be isolated from other tissues. One of these cells is adipose-derived stem cells(ASCs), which are similar to mesenchymal stem cells derived from bone marrow, but can be easily harvested with high yield, which makes adipose tissue an ideal source of multipotent stem cells (Simonacci et al. 2017). Similarly, dental pulp stem cells (DPSCs) have recently received attention due to their readily

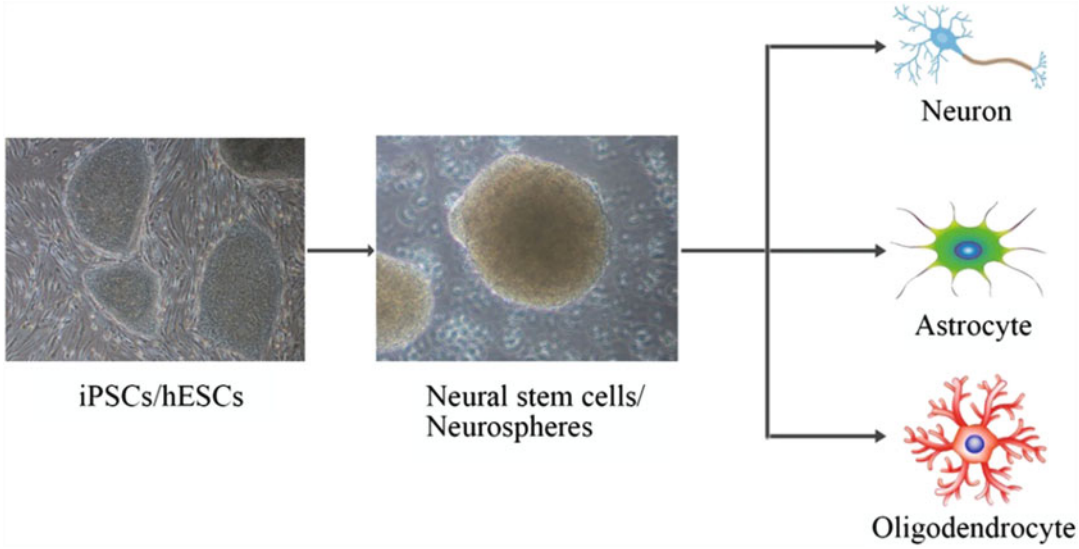

Fig. 1.2 Derivation and differentiation of neural stem cells from iPSCs/hESCs. Human iPSCs/hESCs formed monolayered clonies (left panel); Neural stem cells formed neurospheres in culture (middle panel); differentiated neurons, astrocytes and oligodendrocytes have specific morphologies (Right panel)

accessible source from adult and children (Anitua et al. 2018; Zhang et al. 2016). Furthermore, multipotent stem cells can be obtained or derived from fetal amniotic fluid, amniotic membranes, or placenta (Trohatou and Roubelakis 2017). Recently we have shown that stem cells from human exfoliated deciduous teeth (SHED) were shown to be effective for the transplantation therapy of PD (Zhang et al. 2018). Since the majority of neurodegenerative diseases have neuronal loss, neuronal replacement therapy using neural stem/progenitor cells might be vital to restore functions of lost neurons. The different stem cell sources for the treatment of NDs are summarized in Fig. 1.3.

In mammalian CNS, different kinds of neurons with defined properties, including their neurotransmission phenotypes, location, and connectivity, perform specific functions to complete the CNS circuit. Since neuronal loss is variable among different neurodegenerative diseases, transplantation of the proper phenotypes of neurons and at the right location is very critical to restore the lost function of degenerated neurons. Some neurodegenerative diseases lose defined phenotypes of neurons at certain locations, such as dopamine neurons in the substantia nigra in Parkinson's disease (Cacabelos 2017), motor neurons and astrocytes in ventral horn in amyotrophic lateral sclerosis (Hardiman et al. 2017), and spinal muscular atrophy (Nash et al. 2016), medium spiny neurons in striatum in Huntington's disease (Rüb et al. 2016), and photoreceptor cells in retinal degeneration (Wert et al. 2014). Other diseases lose multiple phenotypes of neurons at different locations, such as different neurons at cerebral cortex and certain subcortical regions in Alzheimer's disease (Akiyama et al. 1989; Mufson et al. 2000). Besides neuronal loss, oligodendrocytic glia are damaged or degenerated in the brain and spinal cord in multiple sclerosis due to destruction of the immune system and other factors that affect the generation of oligodendrocytic glia (Huang et al. 2017). The replacement of well-known phenotypes of neurons at defined locations by transplantation of pre-differentiated neuronal progenitor cells or young neurons of similar phenotypes seems to have more direct therapeutic benefits than the replacement of multiple neuronal phenotypes at diverse locations (Fig. 1.1).

Besides transplantation of exogenous neural stem/progenitor cells for neurodegenerative

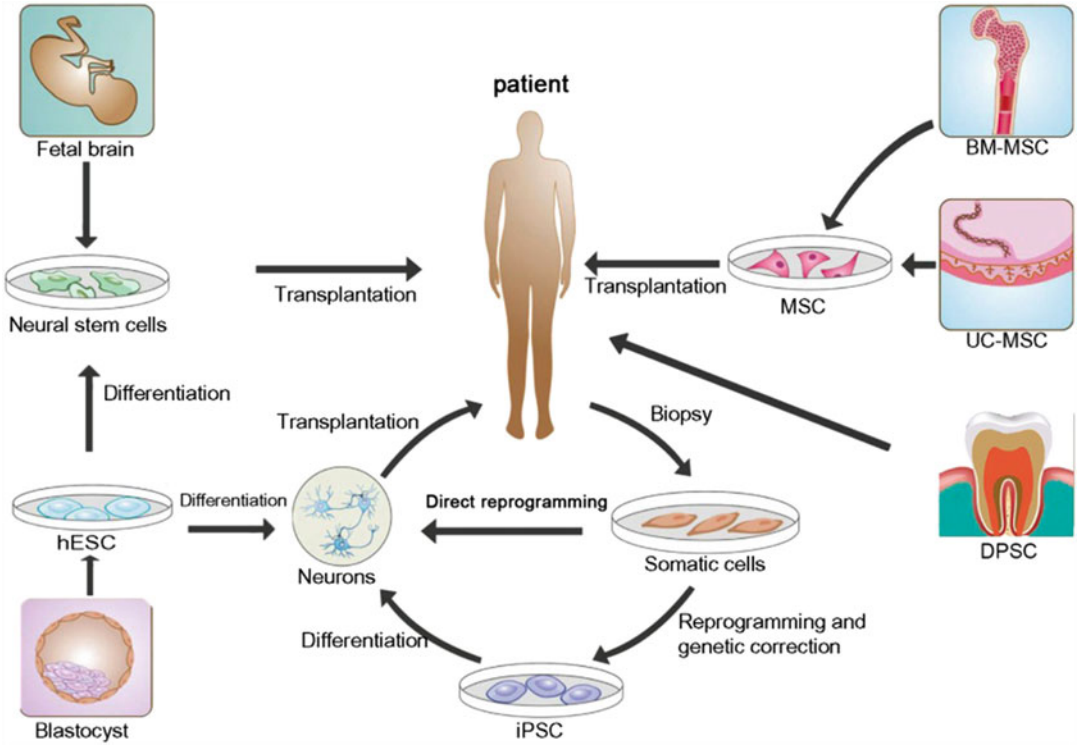

Fig. 1.3 The stem cell sources for transplantation therapy in neurodegenerative diseases: hESCs isolated from the inner cell mass of a blastocyst; neural stem cells from fetal brain tissues; BM-MSC from adult bone marrow; UC-MSC from umbilical cord of the newborn; DPSC isolated from dental pulp tissues of adult or children; and iPSC derived from the reprogrammed somatic cells. (Reproduced from Han et al. 2015b with permission (Review paper))

diseases, new neurons could be regenerated from the brain neurogenic niches, such as subventricular zone and dentate gyrus in hippocampus (Grade and Götz 2017). In addition, neuronal reprogramming that can convert local glia into functional neurons although high efficiency reprogramming in vivo is still a challenge (Gascón et al. 2017; Dorit Trudler and Stuart A Lipton 2019). These additional endogenous approaches have certain advantages over the transplantation approach, such as easy integration to the existed CNS circuit and lack of immune rejection.

Although neuronal replacement therapy is the ultimate goal that could cure neurodegenerative diseases, transplantation of non-neural stem/progenitor cells, such as bone marrow stem cells, could protect existing healthy neurons and glia and even promote neuronal regeneration. This neuroprotection effect is very important to prevent or slow down neurodegeneration, especially at the onset or early stages of neurodegeneration. For example, mononuclear stem cells from bone marrow, including hematopoietic stem cells (HSCs), could completely replace abnormal immune system in an autoimmune disease such as multiple sclerosis (MS) (Abi Chahine et al. 2016).

Similarly, the popular mesenchymal stem cells (MSCs) from bone marrow and other resources are very attractive since they also possess immune modulatory property and immunologically privilege phenotype (Volkman and Offen 2017). In addition, MSCs exhibit other clinically interesting properties, such as augmented growth factor support of degenerated neurons, enhancing endogenous neurogenesis, promoting axonal regeneration, and anti-apoptosis. Furthermore, these non-neural stem/progenitor cells can be genetically modified to over-express neurotrophic

factors for the enhancement of neuroprotection (Uccelli et al. 2011).

Stem cell transplantation approach appears very attractive for future clinical treatments of neurodegenerative diseases. The mechanism behind this approach is neural cell replacement, especially neuronal replacement, as well as neuroprotective and neurorestorative effects. Although most early researchers focus on transplantation of non-neural stem/progenitor cells, such as bone marrow stem cells, due to their easy access for isolation and expansion in culture and certain features for neuroprotection and regeneration, transplantation of neural stem/progenitor cells become more popular since they can replace degenerated neurons and may potentially completely restore functions. The recently developed new techniques greatly expand our ability to generate enriched specific phenotypes of neuronal progenitors or young neurons from a variety of resources in vitro (Livesey et al. 2016; Lu et al. 2016; Vadodaria et al. 2016; Li and Rosser 2017; Xu et al. 2017; Zhang et al. 2018). This will in turn facilitate neural stem/progenitor cell transplant research and potential treatment for neurodegenerative diseases.

4 Future Prospect

The current use of stem cell therapy for NDs faces two major problems: one problem is the immune rejection of the transplanted cells. One major advantage of iPSCs is that these cells can be generated from prospective patients themselves as autologous cells and can provide a sufficient quantity of specialized cells. Thus iPSCs appear to be promising for the production of numerous selective neuronal types for transplantation therapy in NDs. The recent clinical advance in using iPSC-differentiated retinal pigment epithelial (RPE) cells for treatment of patients with age-related macular degeneration has promoted the the clinical trials of iPSC-derived neural cells for PD and other NDs (Mandai et al. 2017; Osborn et al. 2020). The second problem is that the degenerating host microenvironment does not favor the exogenous grafted cells to integrate into the host brains. In order to increase the therapeutic efficacy of transplanted cells, some supporting matrix or small molecules or growth factors should be combined with the cells to improve the host microenvironment for transplanted cells to play functions (Han et al. 2018; Lu et al. 2012). In addition, one or more specialized neural cells are needed for different NDs, indicating that high efficient neuronal differentiation protocols are required to produce more purified neuronal cells from iPS cells. At last the quality and the safety of these cells should be considered before use in human trials. Thus all other chapters of the book will focus the accomplishments made until recently and the challenges remained for the production and use of stem cells or specialized neural cells for treatment of neurodegenerative diseases including PD, AD, ALS, MS, SMA, and retinal degeneration.

Acknowledgments This work was supported by National Natural Science Foundation of China (NSFC 81571241), Department of Science and technology of Shandong Province, China (2017GSF18104), and Basic Research fund from Natural Science Foundation of Shandong Province, China (ZR2019ZD39). Merit Review Award Number I01 RX002264-01A2 from the United States (U.S.) And Department of Veterans Affairs Rehabilitation Research and Development (RR&D) Service and NIH (R21 NS103074-01). We also thank Wei Wang, Nan Zhang, Xianjie Lu, Jing Duan, Na Song, Yan Wang, Li Xu and Yanming Liu in our lab for their technically editing the references and making figures of all the manuscripts.

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Quality Standards of Stem Cell Sources for Clinical Treatment of Neurodegenerative Diseases

2

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1 Introduction

Cell transplantation has been shown to be an effective means of treatment for neurodegenerative diseases or traumatic neural damages. The standardization of clinical cell therapy procedures will help to optimize the efficacy and safety of cell therapy. GMP guidelines for these procedures should be formed by research scientists, clinicians, and administrators and should cover the fields including cell quality, cell dosage, transplantation methods, efficacy and safety evaluations, repeated treatments, and other key policy issues.

In the past two decades, several cell sources have been explored for the treatment of neurodegenerative diseases. These cells include mesenchymal stem cells (MSCs) from both bone marrow (BM-MSCs) and umbilical cords (UC-MSCs), neural stem/precursor cells (NSCs/NPCs) from fetal brain tissues, human embryonic stem cells (hESCs), and induced pluripotent stem cells (iPSCs) reprogrammed from the somatic cells. More details will be discussed on using these cells for the treatment of neurodegenerative diseases in animal models and clinical trials.

To ensure the safety, effectiveness, and replicability of stem cell application for clinical neurorestoration, the Chinese Association of Neurorestoratology suggested initial standards for the culture and quality control of stem cells used for neurorestorative clinical applications (Chinese Branch of International Association of Neurorestoratology (IANR) and Academic Committee of Chinese Association of Neurorestoratology 2012; Huang et al. 2018). Several other study groups also proposed cell therapy criteria for a variety of neurodegenerative diseases (Cooper et al. 2012; Hodges et al. 2007). Based on these guidelines, we proposed that the following stem cell transplantation guidelines which should be considered during clinical applications: cell quality control, cell dosage, efficacy and safety control, standardized procedures for operators to use materials and equipment as well as other related issues. The general guidelines for clinical application of cell therapies by the International Society for Stem Cell Research can also be used for

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F. Han, P. Lu (eds.), *Stem Cell-based Therapy for Neurodegenerative Diseases*, Advances in Experimental Medicine and Biology 1266, https://doi.org/10.1007/978-981-15-4370-8_2

cell transplantation in neurodegenerative diseases (Daley et al. 2016).

2 Cell Quality Control

Quality control management is involved in all processes to handle cells including isolation, culturing and passaging of the cells, cell storage, transportation, and cell transplantation. It also includes the assessment of biological and therapeutical effects as well as other administrative guidelines. All these procedures should be performed in GMP sterile environments. Since detecting microorganisms may take several days or even weeks for characterization, the sterility of the cell preparation must be carefully assessed and maintained.

2.1 Cryopreservation of Clinical-Grade Stem Cells

If cells are actively growing and are not to be used immediately for clinical transplantation, they can be cryopreserved once they reach the 80–90 confluency in cultured dish. The cells should be trypsinized by adding 2 mL of TRYPLE or 0.05% trypsin-EDTA to each T75-flask. Cell digestion should then be ceased by adding 2 mL of culture medium. The cells are then to be collected and cryopreserved accordingly after cells are centrifuged at 1000 rpm for 5 min. The cells are resuspended in 1 mL of frozing medium in each 1.5–2.0 mL vials. In order to maintain accurate storage records, the total cell number of the cryopreserved vials should be marked on the vials. The cryopreservation process should follow the programmed gradient temperature starting by placing the cells at 4 ° C for 45 min, −20 ° C for 1 h, and then −80 ° C overnight. At last the frozen cells are transferred into a liquid nitrogen tank for long-term storage.

2.2 Recovery Procedures of Clinical-Grade Stem Cells

To resuspend the cells for clinical use, the frozen cell storage tube should be carefully removed from the liquid nitrogen and immersed into a water bath (25.0–37.0 °C) to thaw the cells as quickly as possible. Excess water on the surface of the tubes should be dried with sterile gauze, and the cell suspension should be all transfered into a sterile 15 mL centrifuge and centrifuged at 1000 rpm for 5 min to remove the supernatant containing DMSO. The cells should then be rinsed with medium three times before they are placed into a new culture flask with serum-free culturing medium.

2.3 Guidelines for Sterility Detection and Administration of the Stem Cells

For clinical purpose, cell viability should be first checked using trypan blue staining or flow cytometry analysis and the cell viability should be more than 95%. Cellular immunophenotype is to be analyzed using phenotype identification or genetic detection by genotyping. No tumor formations should be found after the cells are transplanted to the patients. The cell products must be reviewed twice, and laboratory personnel should be responsible for performing the quality review and testing. A standardized quality control form is to be signed before the cells are used for clinical patients (Ren et al. 2008). The appropriate cell dosage should be calculated based on the body weight, and usually at least 1×10^7 cells are transplanted to the adults with neurodegenarative diseases. The detailed dosage can follow the clinical cell therapy guidelines for neurorestoration (Huang et al. 2018; Jiang et al. 2011). The temperature and time for clinical-grade cell transportation should be strictly kept at 4 °C in a biosafety transport box and be applied to patients within 2 h after preparation. These quality standards are generally required for the preparation and clinical use of the cells for the treatment of neurological diseases and will be further improved according to the progress of preclinical and clinical trials.

The detection of any exogenous bacteria, fungi, mycoplasma, or endotoxins in the cells is refer to the related requirements. Some quality requirements are summarized in Table 2.1. Specific details are discussed below for using MSCs,

Table 2.1 Some important quality control issues for cell therapy

Safety issue	Criteria
Sterility	Maintain and ensure sterile conditions during the entire preparation and delivery of differentiated cells from MSCs/NPCs/ESCs/iPSCs Check cell batches for microorganism contamination throughout the preparation process
Identity and Purity	Cells should contain a high percentage of cells with specific neural identity and a low percentage of non-reqiured cells Require continuous re-evaluation
Misdifferentiation	Check cells for the presence of any undifferentiated hESC and iPSC which are not allowed in the transplanted cells
Tumorigenicity	Transplant differentiated cells from MSCs/NPCs /ESCs/iPSCs from the original cell bank into nude mice for testing their potential tumorigenicity
Immunogenicity	Perform genotyping to characterize the cells
Viability	Using trypan blue staining to ensure cell viability should reach more than 95%

NPCs, ESCs as well as iPSCs for the treatment of related diseases in clinical trials.

2.4 Quality Control for Human Mesenchymal Stem Cells (MSCs)

MSCs are fibroblastic-like cells, which originate in the early development of the mesoderm and can be derived from different tissues such as bone marrow, the umbilical cord, dental pulp, adipose, and other tissues in newborns and adults. MSCs are multipotent cells and are able to differentiate into various types of tissues, including bone, cartilage, fat, and neural tissues under specific induction conditions. MSCs were defined by the International Society for Cellular Therapy (ISCT) and include the following criteria (Dominici et al. 2006). First of all, MSCs must be plastic-adherent when maintained in standard culture conditions. Secondly, MSCs must positively express CD105, CD73, and CD90 and be negative for CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR surface molecules. Thirdly, MSCs must have the capacity to differentiate into osteoblasts, adipocytes, and chondroblasts in vitro. In 2016, the ISCT further issued the minimal identification criteria for MSCs (Galipeau et al. 2016). A large number of studies have shown that in addition to differentiation to mesoderm tissues, MSCs can play unique neurorestorative effects on neurological disorders and traumatic injuries of the central nervous systems in both animal models and human clinical trials (Chen et al. 2014; Li et al. 2014, 2015; Wang et al. 2013, 2015; Xi et al. 2013).

For the therapeutic purpose of the neurological disorders, umbilical cord MSCs should be isolated from the fetal umbilical cords of normal births or induced abortion of fetuses without brain abnormalities. The derivation process of umbilical cords should be approved by the ethics committee of relevant hospitals or health institutions. Donors need to be excluded for history of inherited diseases and infectious diseases such as HIV and hepatitis B/hepatitis C. After consenting to the donation, the female donors should sign an informed consent form willing to donate their umbilical cords. The freshly isolated umbilical cords should be kept in a sterile bag, then placed in a freezer, and transported to the laboratory as soon as possible. The culture medium of MSCs usually consist of DMEM/F12 with low glucose (1 g/l), 200 mM glutamine, 10% fetal bovine serum (FBS), non-essential amino acid (NEAA), P/S (Penicillin-Streptomycin). In recent years, human platelet lysate (HPL) has been regarded as an acceptable alternative to FBS for MSC expansion (Schallmoser et al. 2007, 2009; Schallmoser and Strunk 2013). All the materials must meet the sterile quality standards without any contaminations of pathogenic microorganisms and endotoxins.

For preparation of umbilical cord MSCs, umbilical cord tissue should be collected into sterile PBS or saline-containing antibiotics.

After being transferred to a clean biosafety cabinet, the umbilical cord tissue should be placed on a sterile culture dish. After stripping off the outer membrane and blood vessels from the surface of the umbilical cords. The umbilical cords are then cut into small clumps using sterile scissors. The chopped pieces of tissues are placed into a 50 mL sterile centrifuge tube and centrifuged at 1000 rpm for 5 min. The resulting supernatant should be discarded, saline-containing antibiotics should be added. The precipitated tissue blocks are thoroughly mixed to loosen them and should be centrifuged again at 1000 rpm for 5 min. Tissue blocks are then to be suspended in DMEM containing 15% fetal bovine serum, fully mixed, and then centrifuged at 1000 rpm for 5 min. The tissue blocks should be precipitated evenly, grown on plastic flasks with poly-lysine–coated surfaces, and then placed in a 5% CO_2 incubator. After the tissue blocks have completely adhered to the flasks, DMEM medium containing 15% fetal bovine serum is added. After cultured for about 14 days, the spindle cells or polygonal cells around the tissue blocks are grown out under an inverted microscope. Over the next week, cells will grow rapidly and relatively uniformly, taking the shape of a long spindle with swirling growth patterns. After the cells cover 80% of the bottom of flask, they should be digested using 0.05% trypsin-EDTA. When the MSCs are passaged to the third to fifth generation, they can be used for clinical applications or cryopreservation.

The phenotypic markers for characterizing MSC are recommended by the ISCT: over 95% of the cells expressing CD73, CD90, and CD105; and 99% of the cells did not express CD34, CD45, and HLA-DR, as measured by flow cytometry (Dominici et al. 2006).

To date, more than 700 clinical trials using hMSCs have been initiated, 40 of which have reached phase III clinical trial. hMSCs have been studied for treatment of CNS disorders including neurodegenerative diseases and acute brain injuries by using intravenous and intrathecal transplanted cells. All reagents and materials for culturing MSCs must be free of exogenous bacteria, fungi, mycoplasma, and endotoxins. Clinical cell dosages can follow the general guidelines for clinical application of cell therapies by the International Society for Stem Cell Research (Daley et al. 2016) and the Chinese clinical cell therapy guidelines for neurorestoration (Chen et al. 2014). An open-label, single-center, phase 1 clinical trial for Alzheimer's Disease (AD) was performed at the Samsung Medical Center in Korea. Allogeneic human umbilical cord blood–derived MSCs were injected into nine patients having probable AD. Three participants were divided into a low-dose group (3×10^6 cells), six others were enrolled into a high-dose (6×10^6 cells) group, and both received bilateral stereotactic injection of human umbilical cord blood–derived MSCs into the hippocampus and precuneus. Symptom improvements were evaluated over 24 months of follow-up as measured by the cognitive subscale of the Alzheimer's disease Assessment Scale (Kim et al. 2015).

2.5 Quality Control for Human Neural Stem/Precursor Cells (NSCs/NPCs)

NSCs/NPCs have the ability to differentiate into neurons, oligodendrocytes and astrocytes, which can provide a large amount of cell sources for transplantation therapy. Studies have shown that the main features of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease are the loss of neurons in specific regions of the brain; therefore, the exogenous neural stem cells could be implanted to achieve significant therapeutic purposes, and these studies have attracted attention from many medical research community (Kallur et al. 2006; Kim et al. 2009; Monni et al. 2014; Studer et al. 1998).

Donors are selected from fetuses aborted at 12-13 weeks of gestation and must have been excluded for brain malformations and history of infectious diseases such as HIV-1, HIV-2, hepatitis B, hepatitis C, and syphilis. Mothers

and their families are informed and required to sign informed consent forms, and the procedure is approved by the hospital ethics committee. Donated samples are kept in a sterile bag in the freezer and are delivered to the laboratory. Fetal brain tissue is isolated and is cultured in complete DMEM:F12 (1:1) medium to include antibiotics, 1% N-2 supplement, 2% B-27 supplement, 20 ng/mL epidermal growth factor, 20 ng/mL fibroblast growth factor, glutamine, and fetal bovine serum. All materials must meet the quality standards for sterile, nonpathogenic microorganisms and endotoxins (Feng et al. 2018).

NPCs isolated from the subventricular zone are to be identified using specific neural antigens including Nestin, neurofilament (NF), glial fibrillary acidic protein (GFAP), and NG2. Immunofluorescence staining or flow cytometry can be used to identify the immunophenotype of NPCs.

Clinical assessment protocols have been modified and the therapeutic effects have been found by detecting behavioral and histological improvement since the first clinical trials transplanting fetal dopaminergic neurons (DA neurons) into Parkinson's disease (PD) patients (Freed et al. 2001; Lindvall et al. 1994). Therapeutic efficacy of transplanted cells was more obvious in younger PD patients, who showed more significant improvements (Ellerstrom et al. 2006; Hagell and Brundin 2001). In general, variable functional outcomes have been shown from the clinical trials, but solid improvements need to be determined by future clinical and imaging evaluations (Barker et al. 2013; Lindvall and Bjorklund 2004). Since transplantation of NPCs in PD patients showed some side effects such as persistent dyskinesia after overnight withdrawal of dopaminergic medication (Olanow et al. 2003), a homogeneous cell population in transplanted tissue might be needed to alleviate dyskinesias symptoms (Politis et al. 2010). In general, DA neuron engraftment cannot be a permanent treatment for PD, follow-up implantations may be required for optimal long-term effectiveness. Overall, clinical trials using NPCs of fetal brains showed the survival of the transplanted cells and some improvements of symptoms in PD patients, while risks of graft rejections should be repressed to increase the number of survived transplanted cells in brains of the patients (Michel-Monigadon et al. 2010).

2.6 Quality Control for Human Embryonic Stem Cells (hESCs)

hESC cell lines were established successfully in 1998 (Thomson et al. 1998). These cells originate from the inner cell mass of the embryonic blastocyst, and are capable of dividing in cultures without differentiating for prolonged periods, and are known to develop into cells and tissues of the three primary germ layers. hESC lines, such as H1, H9, MA09, and I6, on which most clinical trials are based, were not initially clinical-grade cell lines. They were derived as research-grade lines and only later were adapted to clinical GMP conditions. hESC lines are now widely used in various basic and clinical studies (Ellerstrom et al. 2006; Galan and Simon 2012; Jacquet et al. 2013; Rajala et al. 2010; Rodin et al. 2014; Stephenson et al. 2012; Tannenbaum et al. 2012).

In cell cultures, the use of serum-free medium will greatly reduce potential risk of animal hazards (Cooper et al. 2012). Therefore serum-free medium should be chosen whenever possible. Although the effectiveness of the cultures will be slightly reduced, it will greatly reduce potential hazards of animal components contained in differenciated neural stem cells from hESCs (Chambers et al. 2009; Fasano et al. 2010).

A screening of the multiple hESC lines for differentiation propensity has become a standard approach in the selection of hESC lines for particular clinical trials. The yield of differentiated cells derived from hESC lines mainly depends on the quality of the cell source and the efficacy of differentiation protocols.

2.7 Quality Controls for Human-Induced Pluripotent Stem Cells (iPSCs)

In 2006, a major technological breakthrough in regenerative medicine was the report of iPSCs generated from somatic mouse fibroblast cells

by four transcription factors: Oct3/4, Sox2, c-Myc, and Klf4 (referred to as Yamanaka factors) (Takahashi and Yamanaka 2006). iPSCs have a similar gene expression profile and developmental potential to embryonic stem cells (ESCs). Currently, the most commonly used method to generate iPS cells is using the transgene-free episomal plasmids (Kerem Fidan et al. 2016). Human iPSC technology holds great promise for human disease modeling, drug discovery, and stem cell–based therapy (Park et al. 2008; Shi et al. 2017).

iPSCs can be identified using Oct4, Sox2, TRA-1-60 and Nanog-immunofluorescence staining. Specific genetic and epigenetic footprints may influence the molecular and functional properties of each human iPSC (hiPSC) clone in drug screening studies. This is particularly important in studies with disease-specific iPSC lines. Now it is possible to repair mutations in disease-specific hiPSC lines using newly discovered relatively high-precision genome editing techniques, such as clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 (Cong et al. 2013; Han et al. 2015).

One of the major advantages of iPSCs over BM-MSCs, fetal NSCs, and hESCs is that it can be generated from the cells of the individuals being treated. The key trait of iPSCs with autologous transplantation minimizes the risk of rejection and enhances their integration into the brain tissues of patients. In addition, the ethical problems of using aborted fetuses as a cell source are also avoided. Under specific culturing condition and factors, the iPSCs can be differentiated into a specific lineage of mature cells. Until now, great efforts have been tried to bring these therapeutic iPS cells to meet clinical GMP standards. Patient-specific iPSCs have also been used in other fields to evaluate the molecular mechanisms of the diseases and individual's response to specific drug treatment in order to determine whether or these drugs are suitable candidates.

3 Suggested Cell Dosages

Cells must be used at an effective dosage. Thus, the cell dosage and injection volume must be determined and controlled based on the evidence of efficacy and safety. Currently, we recommend the following guidelines: the maximum injection volume of cell suspensions does not exceed 200 mL per injection into brain parenchyma (Chen et al. 2010; Nelson et al. 2002; Savitz et al. 2005), 25 mL per injection into spinal cord parenchyma (Huang et al. 2003; Huang et al. 2008), 10 mL for intrathecal injection into cerebrospinal fluid (Mehta et al. 2008; Sykova et al. 2017), or 10–100 mL for intravenous and intra-arterial routes (Battistella et al. 2011; Friedrich et al. 2012; Mehta et al. 2008). The volume or number of cells being transplanted will be reformulated if further trials show that suitable dose is needed in terms of patient body weight.

4 Efficacy and Safety Control

Efficacy evaluations of cell transplantation should contain validated and established standards or scales currently used in the international community to assess the patients' functional improvements after cell transplantation. Safety evaluations should contain detailed records for cell therapy–related adverse effects such as high fever, headache, nausea, vomiting, anorexia, infection, rash, poor wound healing, dyspnea, increased/decreased blood pressure, increased/decreased heart rate, neurological deterioration, cerebrospinal fluid leakage, seizure, and so on.

5 Standardized Training and Management

A senior scientist/manager should be responsible for the full cell culture laboratory. Operators should possess a cell biology education background and have professional training in cell culture techniques and quality control management for cell-based therapy. Basic standardized training schemes include informational instruction, familiarity with applicable rules and regulations, and skill development. Training methods include centralized teaching, workshops, demonstration, symposiums, and seminars. The training

objectives are to master cell culture techniques, professional knowledge, and quality control processes for managing clinical-grade cells. A standardized management system should be used, to include organizing original work records and data, compliance with current Good Manufacturing Practice protocols, and completion of the laboratory operating procedures.

6 Patient-Informed Consent

Two types of informed consent must be obtained for related studies. The first comes from donors or parents who must give consent for the cells to be used to treat patients. For example, if cells are obtained from aborted fetuses, the parent must understand what the cells will be used for and fill the informed consent form. The second is informed consent of the cells' recipients. Patients and their families have the right to know all of the possible benefits and potential risks of related to the cell transplantation and procedures. Physicians should continue to learn the latest cell therapy–related regulations in order to give objective answers and explanations on relevant subjects. All participants must complete and sign consent forms that have been approved by the appropriate institutional review board or ethics committee before the cell therapy can be performed on patients.

7 Contradications for Undergoing Cell Therapy

Patients with poor health or major organ dysfunction may not be able to withstand surgery or cell therapy procedures. The presence of infections, frequent bleeding, coagulation disorders, and emotional disturbances likewise may introduce undesirable complications. Patients with active neoplastic diseases, hypersensitivity, or pregnancy likewise should be excluded from cell therapy, unless the given therapy is specifically intended to treat these conditions. Clinicians should consider the likelihood that a high incidence of complications risks can lead to unnecessarily negative and undesirable issues for cell therapy or transplantation procedures.

8 Conclusion and Future Research Directions

Cell-based therapy holds clinical potential for the treatment of many neurodegenerative disorders, including AD and PD. In spite of the significant challenges, hESC- and iPSC-based cell therapies have wide clinical applications in treating degenerative diseases, and a large volume of animal studies indicated that iPS cell therapy is considered to be a major treatment for neurodegenerative diseases in the future. To fully realize the potential of these treatments, the preparation of clinical-grade stem cells is a necessary step, and it is necessary to first demonstrate their safety before taking advantage of their efficacy. Initial data from clinical trials using hESC or iPSC-derived neural stem cells to treat PD and retinal degenerations are promising, and these trials are likely to lead to new trials for other diseases. However, for iPS cells to continue their journey toward meeting clinical needs, they must overcome many technical challenges, such as issues of gene mutations, low levels of differenciation efficiency, and differences between batches in the process of amplification. The use of BM-MSCs, fetal NSCs, and hESCs faces an array of safety and ethical concerns. Therefore, a relevant therapeutic progenitor or mature cell type may be identified and grafted in such treatments, providing patients with iPSC-derived neurons or iPSC-derived NSCs as future treatment options.

Acknowledgments This work was supported by National Natural Science Foundation of China (NSFC 81571241), Department of Science and technology of Shandong Province, China (2017GSF18104), Science and Technology Innovation Committee of Shenzhen Municipality, China (JCYJ201803051642562) and Natural Science Foundation of Shandong Province, China (ZR2019ZD39). We also thank Qingfa Chen and

Mengpeng Li in our lab for technically editing the references of the manuscript.

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Stem Cell Therapy for Parkinson's Disease

3

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1 Introduction

Parkinson's disease (PD) is caused by eventual neurodegeneration of dopamine neurons in the substantia nigra of middle brain. It is clinically characterized by bradykinesia, tremor, rigidity, and later postural instability of motor symptoms and related non-motor symptoms. Currently there is no treatment to stop the disease progression of PD. However, over the past 30 years studies indicated that PD is mainly caused by synuclein-mediated protein aggregation (lewy body), leading to cell death of dopamine neurons (DA neurons). Further studies have discovered that other neurons such as the cholinergic neurons or astrocytes may also be affected to induce non-motor symptoms such as depression, constipation, pain, and sleeping dysfunctions in the early development of PD. The most effective treatment is to use levodopa/carbidopa, dopamine agonists (both ergot and non-ergot types) to replace dopamine. Neverthless, the levodopa replacement can only relieve the symptoms of PD without affecting the progression of disease. Furthermore, the levodopa replacement can cause the side effect of involuntary muscle movements called dyskinesias. Another useful treatment for PD is called deep brain stimulation (DBS) which is to surgically implant the electrodes to the subthalamic nucleus to improve motor symptoms of PD by the unknown electrophysiological mechanisms. Most possibly the DBS is to increase the dopamine release of the undegenerated DA neurons, but DBS treatment has been found to lose effectiveness over time. Because of the specific cell death of dopamine neurons in PD, the neural stem cell transplantation has been considered the potential treatment and extensively explored for more than two decades (Berg et al. 2014; Olanow et al. 2001; Postuma et al. 2015).

In the 1980s and 1990s, early studies ever got enthusiastic results by transplanting fetal brain-derived neural stem cells (fNSCs) into the striatums of PD patients. Afterward, the expanded large-scale double-blind studies were tried to use fNSCs for PD patients in different clinical trials. However these studies did not convince the early findings, promoting research to explore other stem cell sources for PD. The bone marrow–derived mesenchymal stem cells (BM-MSCs) or umbilical cord–derived mesenchymal stem cells (UC-MSCs) were also transplanted for the

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F. Han, P. Lu (eds.), *Stem Cell-based Therapy for Neurodegenerative Diseases*, Advances in Experimental Medicine and Biology 1266, https://doi.org/10.1007/978-981-15-4370-8_3

treatment of PD in animal models and got some therapeutic effects. After the successful isolation and culturing of human embryonic stem cells (hESCs) from in vitro fertilized blastocysts, researchers have developed efficient differentiation methods to largely produce dopaminergic progenitors or dopamine neurons for transplantation therapy of PD and other neurodegenerative diseases. To overcome the ethical issues and immune rejection of fNSCs and hESCs, induced pluripotent stem cells (iPSCs) reprogrammed from patients' somatic fibroblasts or blood cells have been recently explored to derive suitable cells for the treatment of PD, even for all other diseases (Han et al. 2015a, b; Lindvall and Björklund 2011; Takahashi et al. 2007).

2 Etiology and Genetic Study

Generally, PD can be divided into familial and sporadic cases. Most of PD cases are sporadic (80–90%) whereas the inherited familial cases only account for 10–20%, which are usually caused by genetic mutations in associated PD genes (Chen et al. 2014; Han et al. 2016; Lesage and Brice 2009). The twin studies also suggested that genetic mutations may not be a major factor causing typical PD, especially for the late-onset patients (Chung et al. 2013; Tanner et al. 1999). However, the discovery of the mutated genes in familial PD has largely contributed to uncover the molecular mechanism and therapeutic targets for PD.

Since the α-synuclein (*SNCA*) gene was first reported as the PD gene in 1997 (Polymeropoulos et al. 1997), more and more genetic linkage and association studies have identified more than 30 genes or susceptible loci related to familial and sporadic PD (Bandres-Ciga et al. 2020). Different mutations in the *SNCA* and *LRRK2* genes of autosomal dominant PD genes have been extensively investigated to explore how the mutant proteins of SNCA and LRRK2 cause the cell death of the neurons and dopamine neurons in the brain. Soon afterward, the PARK2 (Parkin), PINK1, PARK7, PLA2G6, and ATP13A2 of autosomal recessive genes were identified and shown to mainly contribute to the pathogenesis of PD through the mitochondrial-lysosome pathways. In the meanwhile several susceptible genes or loci of Tau, Nurr1, and GBA were also reported to be associated with PD (Han et al. 2016; Yu et al. 2015). Recently more new PD loci (ACMSD, STK39, MCCC1/LAMP3, SYT11, and CCDC62/HIP1R) were identified through the genome-wide association study (Deng et al. 2018; Nalls et al. 2011).

The most important progress of the molecular pathological studies is to recognize the aggregation of alpha-synuclein to be the key factor for PD cases. A lot of studies have identified different point mutations (A53T, A30P, E46K) of SNCA and its genomic rearrangements including the duplication and triplications in different families with PD. It is now understood that the specific pathological lewy bodies in brains of PD patients are mainly composed of the misfolding and aggregation of α-synuclein, ubiquintin, and other proteins in the dopamine neurons (Surmeier 2018). In addition, another autosomal dominant PD gene of *LRRK2* was found to modify the alpha-synuclein and combine to contribute to the pathology of PD. The mutations in *LRRK2* occur in each exon and exon-intron boundaries of the *LRRK2* gene. The most common pathological *LRRK2* mutations are R1441C, Y1699C, G2019A, and I2020T. It was reported that the G2019S and R1441C mutations of *LRRK2* account for 1–3% of familial PD cases and sporadic PD cases. These mutations were shown to increase the kinase activity (Gain of function) of LRRK2 protein to play a toxic role in PD (Grimes et al. 2007; Martin et al. 2014). The parkin gene is the second one to be identified in autosome recessive families of PD (PARK2). Most of the parkin mutations are exonic deletions but missense, nonsense mutations, and genomic rearrangements were also found in PD families (Sliter et al. 2018). Molecular studies have found that the parkin has the enzymatic activity domain of ubiquitin and the RING-like structures with some ubiquitin-ligase activity.

Other susceptible genes are also reported to be associated with PD. We had ever screened 202 familial and sporadic PD patients for

NURR1 (NR4A2) mutations and identified a novel missense mutation in exon 3 of the NURR1 gene in one sporadic PD individual. This point mutation produced a truncated NURR1 protein which is unable to bind the promoter region of the tyrosine hydroxylase (TH) (Grimes et al. 2006). Pathological mutations in GBA were first reported in patients with lysosome diseases such as GD (gaucher's disease). Later GBA mutations were also reported to confer more risk to the development of PD. We have recently performed a case control study in a Chinese cohort with PD and a Chinese control cohort by sequencing all the 12 exons of the GBA gene and found the PD patients have significantly higher frequency of mutations in the GBA gene. Totally we found 9 reported and 3 novel GBA mutations in 184 Chinese patients. These novel mutations are 5-bp deletion (c.334_338delCAGAA), L264I and L314V and the nine known GBA mutations are R163Q, F213I, E326K, S364S, F347L, V375L, L444P, RecNciI, and Q497R. Importantly we identified the novel 5-bp deletion (CAGAA) which produces a non-functional GBA protein of 142 amino acids, which loses major enzymatic function domains of the full GBA protein (Yu et al. 2015). The mechanism for mutations in GBA to cause the neural death of dopaminergic neurons is discussed in the following Sect. 3.2.

3 Animal Models and Molecular Mechanisms

3.1 Animal Model of PD

The cell degeneration of DA neurons is usually accompanied by lewy bodies formed by insoluble aggregates of alpha-synuclein, ubiquitin, and other misfolded proteins, and aggregated lewy bodies are toxic to neural stem cells, neurons, and glial cells (Dawson et al. 2010; Surmeier 2018). To explore the pathogenesis and effective treatment of PD, animal models from different species have been generated to recapitulate the phenotypes of PD. However, each of the current PD models has its limitations and no animal models can completely recapitulate clinical and pathologic characteristics of PD. Currently, three kinds of PD animal models can be available, the neurotoxin-induced model, the transgenic model, and the newly synuclein protein–induced model.

The commonly used drug-induced animal models of PD include mouse, rat, and non-human primate models. The classic PD model is 6-OHDA (6-hydroxydopamine)-lesioned acute PD rat model which can have the motor behavior deficits, but pathologically the 6-OHDA-induced rats do not have pathological lewy body aggregation in dopamine neurons of the brain. 6-OHDA is an analogue of dopamine which can be directly injected to substantia nigra of the rat brains to destroy the DA neurons to induce the hemi-Parkinsonism (Chao Chen et al. 2016). MPTP (1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine) is a synthetic heroin analogue, which can also induce the injury to DA neurons in mice and non-human primates, but not in rats, probably because the rats resist the metabolites of MPTP. MPTP-induced primate monkey models of PD are able to reproduce most, although not all, of the clinical and pathological hallmarks of PD and have been widely used to understand disease pathophysiology and develop potential therapeutics including cell transplantation therapy (Fox and Brotchie 2010; Kikuchi et al. 2017). The transgenic PD models were also developed by overexpressing or knockout the PD-related genes such as LRRK2, SNCA, DJ1, and Parkin. The alpha-synuclein transgenic mice have been developed by overexpressing the wild-type and mutant SNCA (WT, A30P, A53T), but these synuclein-based animal models rarely have the phenotypic characteristics of PD as these models do not have progressive loss of DA neurons in brains of the mice (Chesselet et al. 2008).

Since the transgenic animal models have limitations to recapitulate the specific loss of dopamine neurons, the synuclein protein–based models were created. One study used rAAV vectors to express wild-type or mutant α-synuclein in the dopaminergic neurons in the substantia nigra of midbrains. The motor defects were seen in this model, which include the apomorphine-induced rotation turns. Other AAV

serotypes such as rAAV2/1, rAAV2/5, rAAV2/6, rAAV2/7, and rAAV2/8 have also been used to express alpha-synuclein in animal models to increase transduction efficiency and expression level of α-synuclein in the brain of rats or mice (Koprich et al. 2010; Lundblad et al. 2012). The chronic degenerated PD model is to inject the α-synuclein-formed fibrils to the substantia nigra or other brain areas of the animals to induce the degeneration of the dopamine neurons. This PD model has the formation of protein-aggregated inclusions which can grow and propagate throughout the neurons of the brain (Volpicelli-Daley et al. 2011). The advantage of α-synuclein fibrils-induced models and rAAV-α-synuclein models are formation of pathological cytoplasmic inclusion and have the slowly progressive loss of dopamine neurons, which are more similar to the pathological process of human PD (Paumier et al. 2015).

3.2 Molecular Pathogenesis of PD

The pathogenesis of PD is attributed to the environmental, genetic and aging factors. In the development of PD, the dopamine neurons seem to be more vulnerable to toxic proteins and misfolded protein aggregates. Under the pathological conditions, the cytoplasmic soluble α-synucleins are misfolded and eventually form oligomers to form fibrils and insoluble protein aggregates, which eventually induce the cell death of dopamine neurons through different molecular pathways such as the ubiqintination-mediated protein degradation and endoplasmic reticulum-Golgi localization of the regulating factors such as RAB1a for vesicular transport in midbrain dopamine neurons. The genetic mutations in PD genes can lead to an increase in the production of missense proteins or affect the functions of the proteins such as the activation of the LRRK2 kinase or decrease the Parkin ubiqintinase activity. In addition, the protein trafficking and degradation can also be affected and convergently lead to increased membrane permeability and degeneration of dopamine neurons in PD (Fares et al. 2014; Gonzalez-Horta 2015). Mutations in β-glucocerebrosidase (GBA) were reported to decrease the activity of glucocerebrosidase and increase the production of the glucosylceramides to affect lysosomal function. Recent studies revealed that GBA are associated with misfolded α-synucleins to cause neuronal death in PD, highlighting GBA as a new therapeutic target for PD (Blanz and Saftig 2016; Yu et al. 2015).

In the past years the research on pathogenesis of PD has made progress on the abnormalities of mitochondria, lysosomal-proteasome, autophagy, and oxidative stress pathways through in vitro and in vivo studies. Autosomal recessive gene mutations in PINK1, parkin, DJ-1have uncovered the importance of mitochondrial dysfunction in PD. Several studies suggested that a crosstalk between lysosomes and mitochondria in the pathogenesis of PD. Autosomal dominant mutant LRRK2 interact with alpha-synuclein to induce abnormal protein aggregations which have been shown to localize to mitochondria and lysosomes, further supporting the mitochondrial and endo-lysosomal dysfunctions play key roles in PD (Plotegher and Duchen 2017; Zhang et al. 2018a). The molecular pathological mechanism in the development of PD is diagramed as in Fig. 3.1 (Martin et al. 2011; Zhang et al. 2018a).

4 Cell Transplantation Therapy in PD

Cell transplantation for PD has been explored for more than 30 years, but no reliable cell line or cell sources are available for the patients until now. However, different stem cell lines have been studied for the treatment of PD in animal models and some clinical patients. Here we discuss each of the stem cell lines for the treatment of PD as outlined in Chaps. 1 and 2.

4.1 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) can be isolated from most of the organs and tissues including bone marrow, muscle, skin, dental pulp,

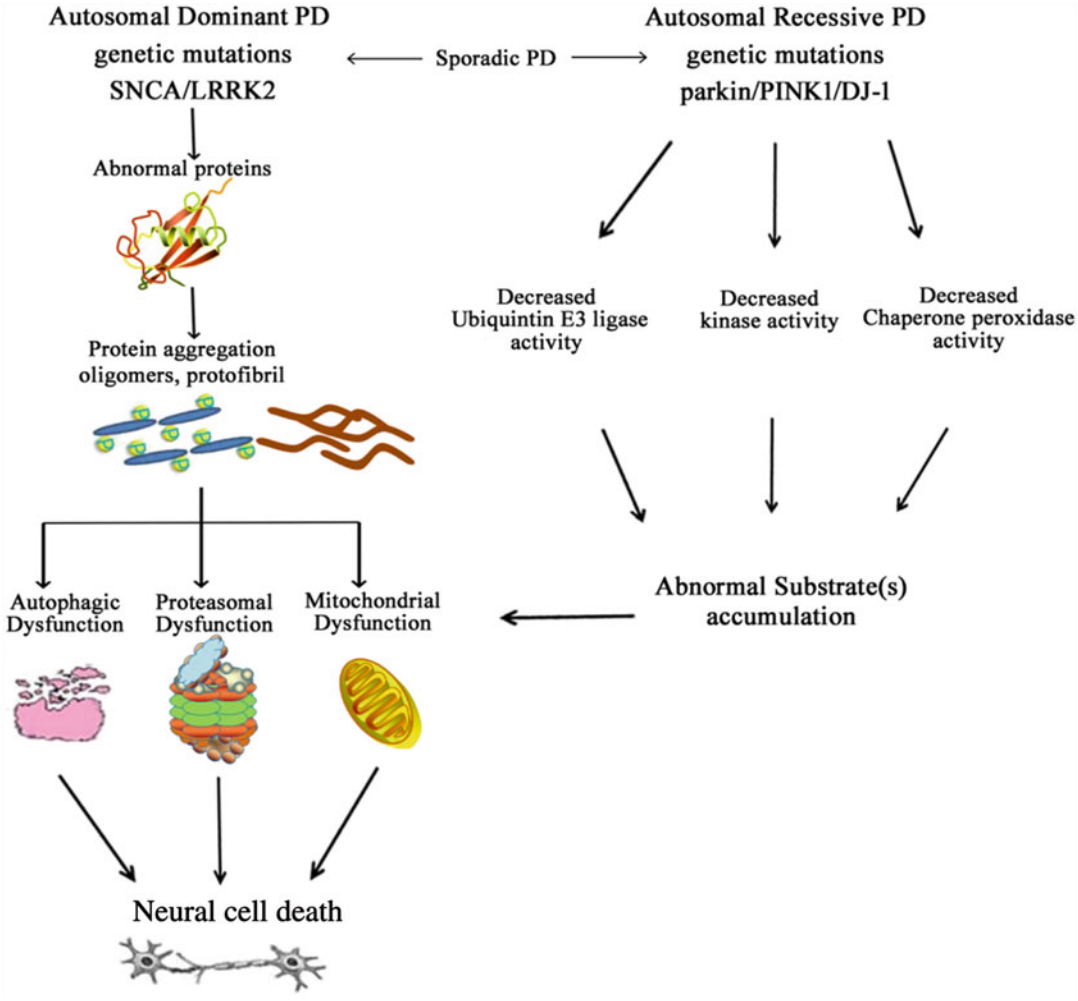

Fig. 3.1 The molecular pathways leading to the dysfunctions of the mitochondria, lysosomal-proteasome and autophagy in development of PD. Autosomal dominant Mutations of SNCA and LRRK2 in sporadic PD lead to protein aggregation and fibrillization to form oligomers and fibrils which disrupt the functions of autophages, lysosomes and mitochondria, which gradually induce neural cell apoptosis in PD. In addition, autosomal recessive loss of function mutations in Parkin, PINK1 and DJ1 impair the functions of Ubiquintin 3 ligase, Kinase and Chaperone peroxidase activities, which lead to accumulation of some unidentified pathogenic substrates and neurodegeneration in PD

peripheral blood, and umbilical cords. These MSCs have been shown to have multipotent differentiation into mesodermal, endothelial, and ectodermal cell lineage including neuronal cells (Hass et al. 2011; Zhang et al. 2016). Mesenchymal stem cells from bone marrow (BM-MSC) and umbilical cords (UC-MSC) have been used in tissue engineering and neural repair because they are easy to be isolated from the adults or newborns and can proliferate for more than 30 passages in vitro without losing their multipotent differentiations. The transplantation of BM-MSCs and UC-MSCs into the 6-OHDA-induced PD rat models was shown to protect damaged DA neurons and differentiate to neural stem cells and mature DA neurons, indicating their therapeutic efficacy for PD. In vitro studies BM-MSCs were able to be induced to neuronal cells expressing markers of neurons (TUJ1) and DA neurons (TH). After transplantation most of

the BM-MSC-derived cells survived in striatum, expressed TH of DA neuronal marker, and restored the motor defects of 6-OHDA-induced rats and MPTP-induced mouse models (Blandini et al. 2010; Han et al. 2018; Park et al. 2008a). As LMX1a is a key transcriptional factor to regulate dopaminergic neuron differentiation, overexpressing LMX1a in BM-MSCs was able to improve BM-MSCs toward dopaminergic differentiation fate, with high expression levels of tyrosine hydroxylase (TH) (Barzilay et al. 2009). Our lab showed that human hUC-MSCs were efficiently induced to convert to neurons (73.1 ± 2.9%) and dopamine neurons (36.3 ± 1.8%) by combinations of noggin, CHIR99021, SHH, FGF8, TGFβ, GDNF, and BDNF. The transplantation of the hUC-MSCs with the growth factors into the 6-OHDA-lesioned rat model of PD was shown to improve motor dysfunctions of these rats from weeks 4 to 16 post-grafting. The efficacy and usefulness of the growth factors in combination with hUC-MSC transplantation in 6-OHDA-lesioned rats provided a promising cell-based treatment strategy for the PD (Han et al. 2018).

4.2 Fetal Brain-Derived Neural Stem Cells and Dopamine Neurons

Fetal brain-derived neural stem cells (NSCs) have been widely studied for their expansion, and differentiation, and transplantation for the treatment of neurological diseases such as PD, AD, ALS, and spinal cord injury (SCI) (Courtois et al. 2010; Kallur et al. 2006). These fNSCs can be isolated from various regions of human fetal brains at 11–13 weeks of gestation and cultured as neurospheres for long-term expansion. The differentiation abilities fNSCs derived from the different brain regions seem to be regionally specific and temporally different from each other. NSCs have a strong proliferative ability and can differentiate into specific neurons, astrocytes, and oligodendrocytes for the treatment of different neurological diseases. The midbrain-derived NSCs are rich in mesencephalic DA neurons suitable for the transplantation treatment of PD (Kim et al. 2006).

After transplantation into the 6-OHDA-lesioned rats, human fetal NSCs were shown to improve the motor defects of these PD rats and the transplanted cells were found to survive in host brains (Monni et al. 2014; Studer et al. 1998). Our lab has recently shown that human fetal NSCs transplanted into the brain of the 6-OHDA-lesioned rats survive and differentiate to dopaminergic neurons in vivo (Wang et al. 2015). Moreover, in PD rats with depleted DA levels, engrafted NSCs tended to be sensitive to microenvironment to differentiate preferentially to DA neurons in the middle brain of the rats (Eriksson et al. 1998; Taupin and Gage 2002). In order to increase the survival of the transplanted cells, some studies modified NSCs to overexpress neurotrophic factors. Some homeodomain proteins of Lmx1a and Msx1 were found to increase the induction of DA neurons. NSCs overexpressing the neural-specific transcription factor ASCL1 were found to increase neurogenesis and to produce larger neurons with more neurites (Kim et al. 2009). Because of the limited availability of the human fetal midbrain tissues, one study developed a method to increase expansion of fetal NSCs and generate more than threefold cells by culturing neurospheres on non-adherent tissue culture plates in neural basal medium containing MEM/F12, HEPES buffer, glutamine, Albumax, and N2 supplement with Shh, FGF8, BDNF, and bFGF. These expanded neurospheres of fetal NSCs can be frozen down for cell banking and retain their ability to efficiently differentiate to the dopamine neurons for the transplantation treatment of PD (Ribeiro et al. 2013).

4.3 Human Embryonic Stem Cells (hESCs)–Derived Neural Stem Cells and Dopamine Neurons

Because of the limited sources of fetal NSCs, human embryonic stem cells (hESCs) have also been explored to derive dopaminergic precursors or dopamine neurons for the cell sources of

PD. hESCs are pluripotent stem cells and can potentially differentiate to any type of tissue cells such as blood cells, cardiac cells, and neurons. At first hESCs can only propagated and cultured on cell feeder layer of mouse embryonic fibroblasts (MEFs) (Thomson et al. 1998). After more than 20 years of research, different protocols have developed to culture the hESCs in feeder-free and xeno-free medium and induce hESCs to dopaminergic precursors or dopamine neurons by specific patterning molecules to regulate midbrain development. After 3–4 weeks of induction, hESC colonies differentiated to tyrosine hydroxylase (TH)–positive dopamine neurons. The transplantation of hESCs-derived neural precursor cells to the 6-OHDA-lesioned rats was shown to alleviate the motor dysfunctions and the grafted cells are able to differentiate to DA neurons in vivo and integrated into the rat striatums (Nakagawa et al. 2014; Zeng et al. 2004; Ma et al. 2011).

To produce specific neurons for PD therapy, other protocols were also developed for generating specifically midbrain-like DA neurons. The treatment of hESC with neural-patterning growth factors SHH and FGF8a resulted in midbrain projection DA neurons with large cell bodies to coexpress DA markers of TH and engrailed 1 (En1) (Yan et al. 2005). These in vitro generated DA neurons were electrophysiologically active and released DA in an activity-dependent manner. After transplantation, these hESC-derived dopamine neurons were shown to integrate in the host brains and were able to improve the motor deficiency of PD rats. To increase the efficiency of DA neuron differentiation, hESCs were induced by adding Noggin and SB431542 to inhibit SMAD signaling. The addition of Noggin and SB431542 induced more than 80% of hESCs converted to neural fate under adherent culture conditions, but the human DA neuron specification is still limited (Chambers et al. 2009). To get more conversion of DA neurons, the same group further developed a midbrain floor-plate-based protocol for generating DA neurons from hESCs by adding activators of sonic hedgehog (SHH) and canonical WNT signaling in differentiation medium. As a result, midbrain floor plate precursors were derived from hESCs by 11 days and by day 25 midbrain DA neurons expressing TH, LMX1A, and FOXA2 were obtained. Importantly these engraftable DA neurons were able to grow for several months in vitro and restored the motor behavior deficits in transplanted Parkinsonian monkeys and 6-OHDA-lesioned rats (Kriks et al. 2011). Another group induced DA neuron conversion of hESCs by expressing LMX1A to obtain more than 60% ventral mesencephalic DA neurons of all neurons derived from LMX1A-modified hESC (Sanchez-Danes et al. 2012). After being transplanted to 6-OHDA-induced PD rats, these hESC-DA neurons integrated into host rats to form the neural connections with the neurons of host brains and improve the motor deficits of PD rats as similar as the transplanted fetal brain DA neurons. This study provided further preclinical basis of hESC-derived dopamine neurons for the treatment of PD patients (Grealish et al. 2014). In the meanwhile mouse embryonic stem cell–derived neurons and dopamine neurons were also shown to differentiate to TH-positive dopamine neurons and played neuroprotective effects on PD (Liu et al. 2013).

4.4 Induced Pluripotent Stem Cells (iPSCs)–Derived Neural Stem Cells or Dopamine Neurons

As NSCs and dopamine neurons from fetal brain tissues and hESCs have ethical and immune rejection problems, in 2006, the successful generation of iPSCs provided great cells for autologous cell-based treatment of diseases by somatic reprogramming technology to introduce Oct3/4, Sox2, c-Myc, and Klf4 into mouse somatic fibroblasts (Takahashi and Yamanaka 2006). One year afterward, patient-specific iPSCs with PD or other diseases were also generated by expressing OCT4, SOX2, c-MYC, KLF4 or OCT4, SOX2, NANOG, and LIN28 in human fibroblasts (Park et al. 2008b; Yu et al. 2007). iPSCs have the same pluripotency with hESCs, overcome the shortages of ESCs, and can be used for exploring the molecular mechanisms, drug

screening, and cell replacement therapy of neurodegenerative diseases such as PD, AD, MS, and ALS (Egawa et al. 2012; Han 2012; Isobe et al. 2015; Li et al. 2011).

To explore the therapeutic effects of iPSCs-derived cells, Wernig et al. reported that iPSCs-derived midbrain-like dopamine neurons, resulting in motor behavioral improvements in rat PD models (Wernig et al. 2008). Because the possibility of integration of viral vectors and transgenes in the genome of iPSCs from patients may induce malignant transformation or affect their differentiation potential, PD patient-iPSCs free of transgenes were derived using Cre-recombinase to excise the reprogramming factors or iPSCs were generated using the non-integrating episomal plasmids to express the genes of OCT4,SOX2, KLF4, NANOG, and tumor-suppressing gene p53 in fibroblasts or blood cells (Kadari et al. 2014; Okita et al. 2011).

After transplantation, the virus-free iPS cells–derived DA neurons were able to survive and improve motor defects of the 6-OHDA-lesioned rats (Hargus et al. 2010). Recently our lab generated iPS cells by retrovirus-mediated expression of OCT4, SOX2, c-MYC, and KLF4 from skin fibroblasts of PD patients and control individuals. We found that the iPSCs carrying the transgenes can also be differentiated to the NSCs and be differentiated to neurons and DA neurons in vitro and in vivo. The grafted iPS cells–derived dopamine neurons integrated to the host brains and significantly alleviated the rotational asymmetry of PD rats (Han et al. 2015b).

As we noticed in transplanted animal models or PD patients, only a small percentage of cells survived whereas a majority of the transplanted are rejected or cannot survive for more than 6 months. This is mainly caused by the immune rejections or no physical integration of the grafted cells to the host neurons to form the functional neural circuit. In order to overcome this issue, we have used the growth cocktail to increase the survival of transplanted cells and improve the host brain microenvironment for the synaptic connections to get the long-term therapeutic effects in PD (Han et al. 2015b; Zhang et al. 2018b) (Fig. 3.2).

To efficiently derive the patient-specific iPSCs, much work has been done to increase the neural and dopaminergic neuronal differentiation of iPSCs. Studer lab reported that using two inhibitors of SMAD signaling, Noggin and SB431542, is able to produce complete neural conversion of >80% of hESC and iPS cells under adherent culture conditions. Hereafter this group modified the induction method to obtain midbrain DA neurons by 25 days and

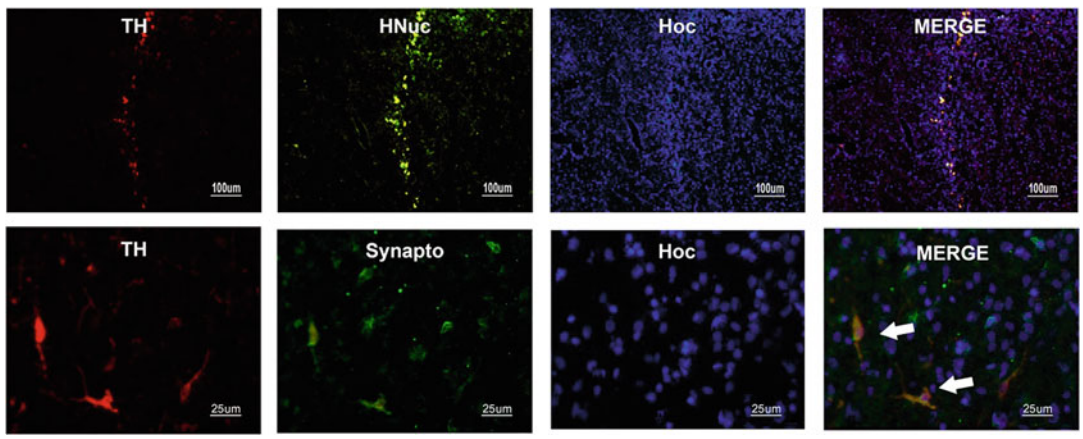

Fig. 3.2 Transplanted human iPSC-derived neural stem cells differentiated to the dopamine neurons and integrated to 6-OHDA-induced rat model with PD. Upper panel: human iPSC-derived dopamine neurons were co-stained with HNuc and TH. Lower panel: Transplanted human iPSC-derived dopamine neurons formed synaptic connection with host brain cells in vivo, which is co-stained with TH and synaptophysin

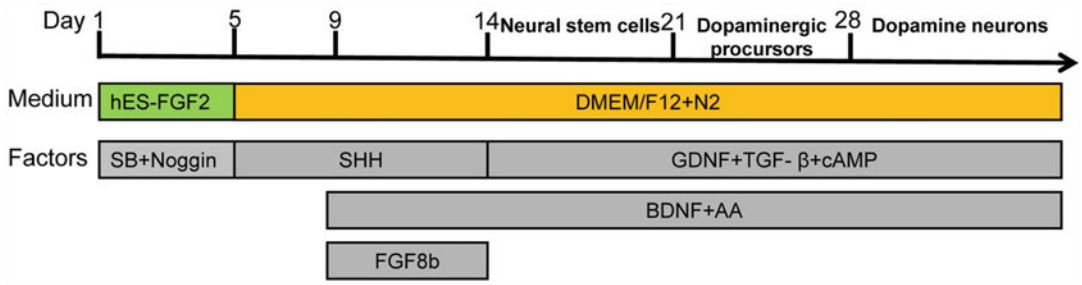

Fig. 3.3 Derivation of dopamine neuron precursors and dopamine neurons from hESCs/iPSCs. The time schedule for 4–6-week dopaminergic neural differentiation includes 4 stages of neural induction (first 2 weeks), neural stem cell (Third week), neuron precursor (Fourth week) and dopamine neuron (5–6 week). hESCs/iPSCs were cultured in hESC medium with SB + Noggin in the first 5 days and after that were cultured in DMEM/F12 + N2 medium with SHH, GDNF, TGF-β, cAMP, BDNF, ascorbic acid (AA), FGF8b at different time points. (Our lab protocol modified from Kriks et al. (2011) and Ma et al. (2011))

transplanted the human ESC-derived DA neurons to the monkey model with PD. These grafted DA neurons survived for a longer time and were able to completely restore the amphetamine-induced rotation behavior and improvements in tests of forelimb use, indicating promise for the development of iPSC-based therapies in Parkinson's disease (Kriks et al. 2011). Other labs used fibroblast growth factor 8 (FGF8) to promote dopaminergic differentiation and sonic hedgehog and GSK3β inhibitor of CHIR99021 to induce the midbrain floor plate (FP) progenitors to establish a robust system for the generation of midbrain dopamine (DA) neurons from human and rhesus monkey embryonic stem cells and induced pluripotent stem cells (PSCs) (Xi et al. 2012). Our lab has also derived the neural stem cells from iPSCs, transplanted the neural stem cells to the 6-OHDA-induced rat PD model, and found the grafted iPSC-NSCs differentiated to the neurons and dopamine neurons in vivo to improve the motor defects of the rats (Wang et al. 2015). The detailed protocol for rapid and efficient conversation of iPSCs to the dopamine neurons is described as in Fig. 3.3.

To produce enough and safe clinical-grade midbrain DA neurons from iPS cells, Isacson et al. have developed a protocol to sort ventral mesencephalic DA neurons from both human ESCs and iPS cells by antibodies of NCAM (+)/CD29 (low) to get rid of the undifferentiated iPS cells. Molecular analysis showed that the sorted neurons were positive for TH, FOXA2, and EN1 and had elevated expression levels of dopamine neuron markers of FOXA2, GIRK2, PITX3, LMX1A, TH, and NURR1. In-vivo studies showed that 16 weeks after transplantation the sorted iPSC-DA neurons were able to form neural connections with the rat brains with TH+/hNCAM+ staining in the host striatum and alleviate motor defects of 6-OHDA-lesioned rats. Their results provided experimental support for the clinical use of iPSC-derived DA neuron in PD (Sundberg et al. 2013). For the clinical purpose of iPSC-derived DA neurons, a transgene-free, xeno-free and scalable differentiation protocol is needed. A suspension culture system was created for the neural differentiation of hESCs and human iPSCs. To decrease the effects of transgenes on pluripotency of iPSCs, several labs developed protocols to use two or three factors to generate iPSCs. iPSCs can be reprogrammed from mouse adult and embryonic fibroblasts with the single factor of OCT4 in combination of small molecules of CHIR 99021,VPA, TGF-β inhibitor (616452) (Li et al. 2011). A recent study also indicated that the derivation of naive iPSCs from rhesus monkey fibroblasts can be obtained with only small molecules, omitting the OCT4, which provided a valuable cell source for further use in disease modeling and pre-clinical study (Fang et al. 2014).

To determine if the human iPS cell–derived DA neurons can survive and play therapeutic

effects in a non-human primate model of PD, a Japanese group transplanted the CORIN-sorted iPSC-dopaminergic progenitor cells to MPTP-induced PD monkeys and found that the grafted cells further differentiated to the DA neurons which extended their neurites into the host striatum and improved the spontaneous movement of the monkeys. These transplanted cell did not produce any tumor cells in the monkey brains for more than 2 years, indicating the safety of human iPS cell–derived dopaminergic progenitor cells to be used for clinical patients (Kikuchi et al. 2017).

To overcome the possibility of tumoric cells restraining in the iPS-dervied DA neurons, another approach is to generate direct dopaminergic precursor cells/dopamine neurons from somatic fibroblasts. A lot of studies have reported the generation of trans-differentiated DA neurons by directly reprogramming the fibroblasts with different combinations of transcription factors such as Mash1 (Ascl1), Nurr1 (Nr4a2), Ngn2, Sox2, Lmx1a, and Pitx3 (Caiazzo et al. 2011; Kim et al. 2014). Since most of these studies used the lentiviral expression of dopaminergic genes to convert fibroblasts to DA-like neurons and have the risk to produce genome instability, recently Mou et al. used silica nanoparticles (MSNs) as a non-viral delivery system to express the key dopaminergic genes of Ascl1, Brn2, and Myt1l to convert mouse fibroblasts (MFs) into functional dopaminergic neuron-like cells. These DA-like neurons were validated to express the DA neuron-specific markers and have electrophysiological properties dopaminergic neurons (Chang et al. 2018). The problems to use the directly converted neurons is that the percentage of directly converted DA neurons from fibroblasts is still low and their purification should be obtained by sorting or other selection methods.

5 Clinical Trials in Using Stem Cells for the Treatment of PD

Following the beneficial therapeutic results of stem cell transplantation on animal models, the first clinical trials for PD patients were carried out in the late 1980s using fetal brain neural stem cells (fNSCs). After that more clinical studies have been done and significant effects were found by examining behavioral and histological improvement of the PD patients with transplanted fetal tissues (Freed et al. 2001; Lindvall et al. 1994). In a patient transplanted with fNSCs, clinical symptoms were shown to be gradually improved and L-dopa treatment could be withdrawn after 6 years of transplantation. After 10 years of grafting, the PET scan showed that patient still had dopamine release from grafted dopaminergic neurons (Piccini et al. 1999). Interestingly a study by Freed et al. compared the therapeutic effects of fNSC transplantation in patients who are younger than 60 years old with the patients who are older than 60 years and showed that more significant improvement of movement symptoms was seen in younger PD patients than the older patients (Freed et al. 2001). This suggested that the host brain microenvironment might play an important role in the treatment efficiency of grafted neural stem cells (Hagell and Brundin 2001). Although some variable improvements were found from the clinical trials, the therapeutic effects were confirmed by clinical examinations and imaging evaluations (Barker et al. 2013; Lindvall and Bjorklund 2004). Some patients have improved so well that L-DOPA could be withdrawn for several years (Olanow et al. 2003; Piccini et al. 1999). However, some cell-transplanted patients developed side effects such as dyskinesia. It was shown that transplanted cells containing serotonin neurons were easier to induce this side effect, suggesting that dyskinesia may be avoided by using purified dopaminergic neurons (Freed et al. 2001; Lindvall 2015; Olanow et al. 2003; Politis et al. 2010).

To know how long transplanted cells survive in PD patients, some clinical studies analyzed the brain slices of post-mortems 16 years after cells were grafted and showed that transplanted fNSCs were able to survive without pathology (Mendez et al. 2008). The transplanted fetal cells in brains of patients were found to stain with DA neuron marker, TH, indicating the transplanted DA neurons survived (Fig. 3.4a) (Mendez et al. 2008). However other two studies reported that

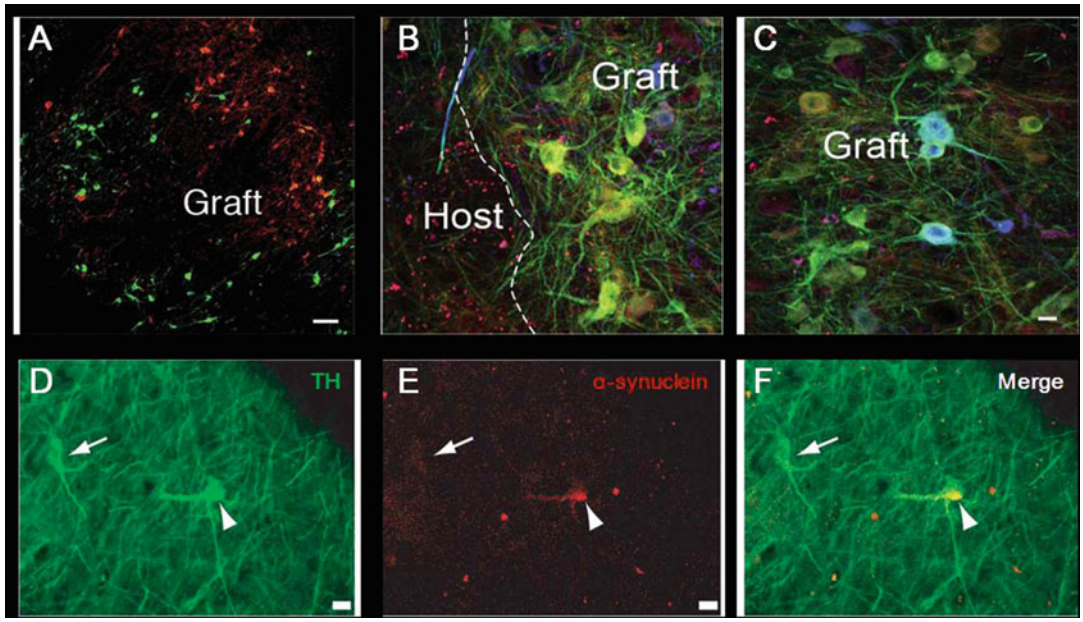

Fig. 3.4 Lewy bodies were formed in grafted cells transplanted to patients with Parkinson's disease. (**a**) Some of the grafted fetal neural stem cells differentiated to the dopamine neurons stained with TH (Red) and serotoninergic neurons stained with TrypOH (Green). (**b**) TH/Girk2-stained dopamine neurons were preferentially located in the peripheral areas of the grafted cells. (**c**) TH/Calbindin-stained dopamine neurons were preferentially located in central areas of the grafted cells. (**d–f**) Double immunolabeling shows colocalization of TH (green, **d**) and a-synuclein (red, **e**) in a graft (arrowheads). One dopaminergic neuron does not contain detectable synuclein (arrows, **d**, **f**). Modified from Mendez et al. (2008) and Li et al. (2008)

alpha-synuclein-positive lewy bodies were also found in the transplanted cells in brains of PD patients, suggesting the pathological process of the host patients affecting the grafted cells. The lewy bodies were stained with alpha-synuclein and ubiquintin, indicating that the important role of synuclein in aggregated proteins of patients. (Kordower et al. 2008; Li et al. 2008). As other allogeneic transplantations, there is also a risk of graft rejection which may affect the efficacy of the transplanted cells (Michel-Monigadon et al. 2010) (Barker et al. 2013). Some of the clinical trials with fetal brain-derived NSCs or dopamine neurons to treat PD are summarized in Table 3.1.

In order to develop uniformed standards to evaluate the therapeutic effects of the transplanted fNSCs, a multicenter and collaborative study of European Union (TRANSEURO) was formed in 2010 to design the guidelines for clinical trials of PD patients with stem cell–based therapy. These screening standards include selection of patients, the time course of their disease (disease duration 2–10 years), and age range of 30–68 years old at the time of inclusion, being responsive to levodopa therapy. These guidelines also contain preparation of cells and location of cells to be transplanted; immunosuppression application after transplantation and time course of follow-up; minimum numbers of patients to be enrolled and clinical assessment standards such as the MRI and PET-CT. The new clinical trial for more than 100 patients suffered with PD has completed in this study, and results are in the analysis (Evans et al. 2012; Moore et al. 2014). A recent clinical study indicated that a patient with transplanted fetal dopamine neurons have significant recovery in striatal dopaminergic function. Pathological examinations showed a graft-derived dopaminergic reinnervation of the putamen can be survived for 24 years with no evidence of immune response in spite of some severe host brain pathology (Li et al. 2016).

Table 3.1 The clinical outcomes of PD patients transplanted with fetal brain-derived neural stem cells

No. of patients transplanted with NSC	Observation time	Symptom improvement 0/0	Side effect of dyskinesia	References and publication year
1	12 months	1/1	No	Lindvall et al. (1990)
6	10–72 months	4/6	No	Wenning et al. (1997)
5	18–24 months	2/5	No	Hagell et al. (1999)
20/40	3 years	17/20	No	Freed et al. (2001)
23/34	24 months	6/23	Yes	Olanow et al. (2003)
2	8 years	2/2	Yes	Pogarell et al. (2006)
5	9–14 years	Not available	Not available	Mendez et al. (2008)
1	14 years	1/1	Yes	Kordower et al. (2008)
2	11–16 years	Not available	Not available	Li et al. (2008)
33	2–4 years	45%	Not available	Ma et al. (2010)
3	13–16 years	Yes	Not available	Politis et al. (2012)
2	18 and 15 years	2/2	Not available	Kefalopoulou et al. (2014)
1	24 years	1/1	No	Li et al. (2016)

Note: 20/40 and 17/20 indicates that 20 of 40 patients and 17 of 20 patients were transplanted with fetal brain NSCs and other patients were in the control group

Overall the fetal brain-derived dopaminergic neurons can survive for as long as 16–24 years in patients' brains and improved the functions in some patients, although the outcomes of other clinical studies are variable. Another problem is the availability of the fetal brain tissues which limited the wide use of fNSCs. To overcome this problem of fNSCs, an international corporation was set up for Gfore in 2014 to start the world's first clinical studies of an iPS cell–derived dopaminergic neuron therapy for PD in Europe, USA, and Japan. Some results of the trials are in the progress. At the same time outside of the Gforce, other clinical trials using iPS cell-DA neurons for the treatment of PD including the Chinese clinical trial for an HLA-matched HESC-derived dopaminergic neuron transplantation therapy for PD (NCT03119636, http://clinicaltrials.gov). Since August 2018, a Japanese group led by Takahashi has started the first clinical trial to use the iPSC-derived dopaminergic precursor cells and the incoming results will soon benefit for the PD patients (Takahashi 2019). The results of clinical trials with HESC- or iPSC-derived DA neurons for treatment of PD will be available soon.

6 Conclusion: Translation to the Clinic

A large number of studies showed that transplantation of fNSCs/hESCs/ iPSCs and their derivatives into animal models has a solid therapeutic effect for PD. One of the major advantages of iPSCs over BM-MSCs, fetal NSCs, and hESCs is that iPSCs can be generated from the autologous cells of the individuals being treated. Once the autologous skin or blood cells are reprogrammed into iPSCs, the iPSCs can be induced to a specific neural lineage such as dopaminergic precursor cells or mature DA neurons (Garitaonandia et al. 2018; Kiskinis and Eggan 2010). Thus, autologous or HLA-matched iPSCs seem to have advantages over other cell sources. Until now, a ton of work has been done to improve the generation, differentiation, and potential clinical applications of iPSCs, especially with great efforts made to bring these therapeutic cells to meet GMP (Good Manufacturing Practice) standards in order to apply the iPSC-derived dopaminergic cells for the treatment of PD and other neurodegenerative diseases. There are several challenges which need to be overcome for clinical uses of iPSCs.

The first challenge is to ensure the efficacy of iPSC-derived dopaminergic neurons after transplantation to the patients. The iPSC-derived dopaminergic neurons have to play similar therapeutic efficacy as human fetal dopaminergic neurons. Since animal research results may not be directly translated to the patients, more clinical trials are needed to define the GMP-graded cells, cell dosages, the injection routes and locations as well as the standard methods to evaluate the neural circuit formation, DA release, and the therapeutic effects of transplanted cells on patients. The second challenge is to ensure the safety of transplanted cells by reducing the tumor formation and the side effects of grafted cells. It is required that the residues of undifferentiated hESCs/iPSCs should be less than 1% to avoid the teratoma formation after transplantation to patients. The patient-derived iPSCs may harbor some mutations in the genes of SNCA, LRRK2, Parkin, Dj-1, GBA, or other loci associated with PD (Lister et al. 2011; Yu et al. 2015). These concerns are related to donor source of iPSCs and the reprogramming approaches which can produce new mutations in iPSCs. To maintain the genomic stability of iPS cells, non-integrating vectors should be used to express the reprogramming genes or combine with small molecules to generate clinical applicable iPSCs (Hou et al. 2013). On the other hand, several gene editing approaches have been developed including the zinc finger nucleases (ZEN), transcription activator-like effector nucleases (TALEN), and CRISPR/Cas9 (Jiaxin Xie et al. 2018; Wang et al. 2016). iPSCs with *LRRK2* G2019S mutation were able to be corrected and the *LRRK2* mutation correction rescued the phenotypes of differentiated neurons (Reinhardt et al. 2013). Soldner et al. reported that the iPSCs with *SNCA* mutation (A53T) was repaired using zinc finger nuclease (ZFN)–mediated nuclease approach and genetic repair of the A53T mutation in the patient-derived iPSCs did not affect their differentiation ability to dopaminergic neurons. A recent study combined the Cas9/CRISPR and piggyBac technologies to generate genome-edited footprint-free human iPSCs at the rate of 10–20%, which will largely increase clinical application in editing the iPSCs derived from PD patients and other patients with genetic mutations (Safari et al. 2019; Wang et al. 2017).

The third challenge is the efficiency of generating iPSCs and the purity of iPSC-derived cells. Since the lower efficiency of fully reprogrammed iPS cells through the non-integrated method is affecting the genome editing and their application, some studies tried to resolve his potential problem by the addition of VPA and other chemicals to increase the generation efficiency of iPSCs (Wang et al. 2011). A recent study summarized the generation of non-integration and feeder-free iPSCs by sendai viral vectors to express OCT4, SOX2, KLF4, and c-MYC in peripheral blood mononuclear cells (PBMCs) and reported the reprogramming efficiency is approximately 0.01% (Ye and Wang 2018). For increasing the purity of iPSC-derived dopaminergic precursor cells/dopamine neurons, several efficient differentiation methods were described in the previous sections. Recent studies purified the human iPS cell–derived dopaminergic neural precursor cells by cell surface-specific marker CORIN to sort the $CORIN^+$ cells. The $CORIN^+$ cells accounted for more than 30% of totally differentiated cells. Importantly these sorted $CORIN^+$ cells expressed the dopaminergic precursor markers of NURR1 and FOXA2. After transplantation to the rat and monkey PD models, these $CORIN^+$ cells differentiated to the dopamine neurons in vivo and improved the locomotive defects of these animal models without tumor formation, ruling out the concerns on tumorigenesis of iPS cell-differentiated cells (Kikuchi et al. 2017; Samata et al. 2016).

The fourth challenge is to screen for the most suitable PD patients to be transplanted with reprogrammed iPS cell–derived dopaminergic neurons in the clinical trials. To achieve the best efficacy and safety, patients have to be in a relatively earlier disease progression stage and have a chance of therapeutic benefit when the dopaminergic neuron loss is mainly affecting the caudate-putamen of midbrain and is not dispused in the forebrains of the patients (Barker et al. 2017).

Acknowledgment This work was supported by National Natural Science Foundation of China (NSFC 81571241), Department of Science and Technology of Shandong Province, China (2017GSF18104), Science and Technology Innovation Committee of Shenzhen Municipality, China (JCYJ201803051642562) and Basic Research Fund from Natural Science Foundation of Shandong Province, China (ZR2019ZD39). We also thank Wei Wang, Jing Duan, Xianjie Lu, Hao Song, Nan Zhang, and Yanming Liu in our lab for technically editing the references and preparing figures of the manuscript.

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Stem Cell Therapy for Alzheimer's Disease

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1 Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease which causes apoptosis of neural cells and dysfunction of synaptic connections in the brain, leading to memory loss and cognitive impairment and decreasing the living quality of patients. It is estimated that AD affects more than five million cases in United States of America, more than six million cases in China, and more than 30 million people worldwide. With the increasing number of aging individuals, more than 3–5% of population over the age of 65 years old are possibly suffering from AD with an estimated 5–7 million new cases of AD each year (Barnes and Yaffe 2011; Holtzman et al. 2011; Rajan et al. 2018; Robinson et al. 2017).

AD is mainly caused by degeneration of neurons throughout the brain and particularly in the areas of basal forebrain, hippocampus, and cortical brain. Pathological studies showed that in the development of AD, mis-folded proteins of Tau and amyloid-β (Aβ) aggregated to affect cholinergic neurons and their synapses and eventually damage other neurons in different regions of the brain. Although some acetylcholinesterase inhibitors can temporarily relieve some symptoms of AD, no effective treatment is available. Recent studies indicated that in addition to the affected neurons, dysfunction of astrocytes and microglial cells has contributed to the early neural degeneration of AD (Boisvert et al. 2018). Thus, stem cell transplantation has great potential to replace the lost neurons and glial cells and increase the endogenous neurogenesis for the treatment of AD.

2 Molecular Pathological Mechanisms of AD

Etiologically AD can be classified into two classes: familial cases of AD (FAD) and sporadic cases of AD (SAD). The FAD patients seem to have early onset under the age of 60 and are only about 5–10% whereas SAD accounts about 90–95% of the AD cases. However FAD and DAD share similar clinical characteristics

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F. Han, P. Lu (eds.), *Stem Cell-based Therapy for Neurodegenerative Diseases*, Advances in Experimental Medicine and Biology 1266, https://doi.org/10.1007/978-981-15-4370-8_4

including progressive memory and cognitive dysfunctions and other neurological defects such as synapse loss and selective degeneration of specific brain regions (Goldstein 2012; Holtzman et al. 2011). Over the past two decades, the causative genes and susceptible genes for FAD and SAD have been identified by molecular genetics studies. The first autosomal mutation in the amyloid precursor protein (APP) gene was reported in 1991. Later, AD was also found to be associated with mutations in presenilin 1(*PS1)* and presenilin 2 (*PS2).* The other AD-associated genes include late-onset gene (apolipoprotein E, ApoE), tau and several potential risk genes (Barbier et al. 2018; Tanzi 2013). The pathological process of SAD is attributed to interaction of different genetic and environmental factors on the neurogenesis. With the advances in whole-exome sequencing (WES) and whole-genome sequencing (WGS) technology, other susceptible genes such as SORL1, HLA-DRB5/DRB1, PTK2B, SLC24A4-RIN3, INPP5D, FERMT2, CASS4, and TRIP4 were also identified to be associated with AD (Chouraki and Seshadri 2014). Accumulated studies have shown that the beta-amyloid (Aβ) plaques and neurofibrillary tangles (NFTs) of tau proteins are the pathological hallmarks of AD (Kuruppu et al. 2016; Nedelsky and Taylor 2019). In the degenerative neurons of AD patients, the Aβ peptide-formed plaques associated with phosphorylated tau protein filaments (tau tangles) eventually induced apoptosis and dysfunction of neuronal cells, glial cells, and synaptic plasticity, indicating the requirement of tau protein for the neurotoxic effects of Aβ deposits. The tau and Aβ peptides are found to be directly interacted to promote aggregation and hyperphosphorylation of tau protein (Moustafa et al. 2018; Rank et al. 2002; Shipton et al. 2011). These Aβ plaques and tau protein–related neurofibrillary tangles eventually spread in almost all brain regions and induce the cytotoxicity and cell death of the neural cells, including neurons, glial cells, and neural stem cells (Fig. 4.1a). In the development of AD, the memory and language dysfunctions of patients are first affected by increased soluble and insoluble Aβ peptides, which are produced from the sequential proteolytic processing of amyloid precursor protein (APP). The Aβ oligomer/polymers are then aggregated to form the Aβ plaques while the formation of tau-related neurofibrillary tangles (NFTs) in the brain induces extensive degeneration of neurons and cell death (Fig. 4.1b). APP is mainly responsible for regulating neurogenesis, synapse formation, axonal transport, and neural plasticity in the neural cells. The cleavage of APP by the enzyme of β-secretase and γ-secretase results in the formation of the Aβ peptide fragments made of the 40 (Aβ40) and 42 (Aβ42) amino acid. The fragments of these Aβ peptides then aggregate to form Aβ-oligomers or Aβ–polymers to produce Aβ plagues in the brains of patients (Thinakaran and Koo 2008). The component of γ-secretase enzyme, such as PS1 and PS2, can also cleave APP to produce the Aβ40 and Aβ42. Besides, PS1 or PS2 regulates autophagy and the endosome/lysosome pathway (Mattson 2004; Mandelkow and Mandelkow 2012; Wang et al. 2013). Since the deposits of Aβ plagues and NFTs are toxic to neurons and glial cells, these cells can have autophagy and programmed apoptosis. Usually cholinergic neurons and their synapses are affected first and then other neurons are involved. The Aβ plaques and neurofibrillary tangles spread to the neurons of other brain regions to induce brain shrink, and the neurons and glial cells gradually degenerate or die, causing the cognitive dysfunction of AD (Brunholz et al. 2012; Gunawardena and Goldstein 2001; Reitz 2012; Walter Gulisano et al 2018).

3 The Animal Models of AD

In order to explore the effective therapy for AD, a large number of animal models have been developed from different species, including C-elegans, drosophila, mouse, rat, and non-human primates. Most of the animal models can only recapitulate some phenotypes of AD. The early-aging animals

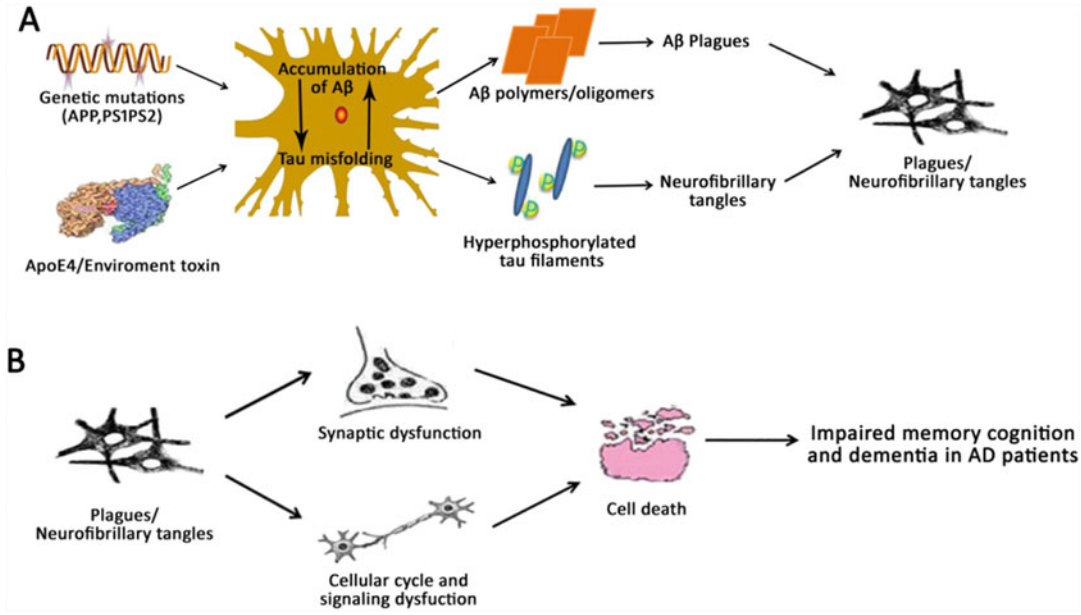

Fig. 4.1 Molecular pathological mechanism of Aβ plagues and NFTs in Alzheimer's disease. (**a**) Aβ plaques are formed through oligomerization and misfolding of Aβ peptides whereas tau-related neurofibrillary tangles (NFTs) are produced by phosphorylation of Tau-protein in brain of AD patients. (**b**) The signal cascades for Aβ plaques and NFTs to induce the neural cell death

can be used as natural AD models, but these models rarely recapitulate the characteristic phenotypes of AD. For drug discovery and stem cell transplantation studies, the commonly used animal models for AD are chemical-induced and transgenic animal models.

3.1 The Chemical-Induced Animal Models

These animal models of AD are usually generated by applying inorganic chemicals or proteins or protein polymers to the specific brain regions such as cortical regions or hippocampus to induce acute or sub-acute injuries to neural cells. Chemical-treated animals must show some impairment of memory and cognitive function similar to some characteristic features of AD. Aβ proteins can be administered to cerebral ventricles to induce the rodent models of AD using a single stereotactic injection, or repetitive injections. Injecting Aβ peptides into the hippocampus of rats or rhesus monkeys can induce some phenotypic abnormalities similar to clinical features of AD, but these animal models did not have progressive deposit of pathological Aβ plaques (Castane et al. 2010; Nakamura et al. 2001; Yamada et al. 2005). Direct intracerebral administration of Aβ peptides was reported to cause learning and memory deficits as well as behavioral alterations similar to AD. Although the exogenous injection of Aβ proteins can produce some neuropathological changes of Aβ plaques in human AD, the full complexity of the human pathology could not be reproduced (Sipos et al. 2007). These chemical-induced animal models can be used to investigate the mechanism of memory loss, motor dysfunction and the neuronal cell death of the lesioned brain regions. The disadvantages of these chemical-lesioned models are to induce lesions of all neural cells in the lesions and cannot follow the progressive pathogenesis in major affected brain regions of AD (Van Dam and De Deyn 2011).

3.2 Transgenic and Knockout Animal Models

Genetic engineering has been widely used to over-express or knockout the related disease genes in animals to understand the detailed molecular pathogenesis of inherited diseases in which causative genes were identified. The first AD transgenic mouse model (Tg2576 mouse line) was generated by over-expressing the *APP* gene and was reported to have a markedly amyloid beta (Aβ) deposit in the hippocampus and cortex of their brains one year after birth. The problem with APP-transgenic mice is that these mouse models failed to produce tau-related NFTs that is fomed in the AD patients' brains (Goedert et al. 2006; Kalback et al. 2002). Another transgenic mouse AD model is generated by over-expressing *PS1*, and it has been shown to have some apoptotic neuronal cell death in the neocortex and hippocampus and have higher levels of amyloid beta-42 (Aβ-42) in the mouse brains, but no abnormal pathological Aβ plaques were formed in the hippocampus and neocortex of these mouse brains (Chui et al. 1999). To combine the advantage of APP and PS1 transgenic mouse models, a double transgenic mouse model was subsequently created from a cross between the APP mouse (Tg2576) and the PS1 mouse (with M146L mutation). It has been shown that a larger number of Aβ deposits are found in the hippocampus and cerebral cortex in these double transgenic mice, which appeared earlier than that in the single APP transgenic Tg2576 mice. Importantly these double transgenic mice clearly had an increased Aβ-42/43 in their brain regions. In the double transgenic mouse, more severe neuronal loss was also seen than that of the single transgenic mice with mutations of *APP* or *PS1* (Rutten et al. 2005). In addition, the Aβ deposits in cerebral cortex and hippocampus were able to be seen compared to those in the control mice (Fig. 4.2). For exploring the process of molecular pathology caused by combined genetic mutations, a triple transgenic mouse model with *APP*, *PS1*, and *PS2* was created and the pathological Aβ deposits were found in the brain regions of these triple transgenic mice. These mice also showed impairment of their synaptic plasticity, such as long-term potentiation and cognitive function. This triple transgenic mouse model offers a great approach to study the pathogenesis and screen for the drug treatment for AD (Du et al. 2008; Oddo et al. 2003).

For the sporadic or late-onset AD cases, no such mouse models exist to completely reproduce the clinical features and late-onset pathological progression. Some transgenic rat models were developed by over-expressing APP and PS1 (Lopez et al. 2004; Nakamura et al. 2001). However the rat models rarely developed the intracellular Aβ accumulation and the phenotypes of cognitive deficits in AD patients (Flood et al. 2009; Vercauteren et al. 2004). A transgenic rat line (McGill-R-Thy1-APP) expressing the double mutated human APP protein was found to have the impairment of cognition and extracellular Aβ deposits in brains of the rats by the age of 6 months (Leon et al. 2010). The fruit-fly Drosophila melanogaster has been widely used as an in vivo animal model to study neurodegenerative diseases including AD (Iijima and Iijima-Ando 2008). The advantage of the fruit-fly model is that the brains of fruit-flies are similar to human brains in several aspects including the structures, gene expressions, cell signaling regulation, membrane trafficking, especially the axonal trafficking, neuronal connectivity, and synaptogenesis (Sang and Jackson 2005). More importantly fruit-fly has a short life cycle to examine the detailed disease progression of AD. In addition, the dysfunction of learning and memory in AD patients can be recapitulated in fruit-fly model, but the cognition function cannot be measured. The zebrafish was originally used to model the development of vertebrate and now has also been used to model the molecular mechanism of human diseases such as AD (Lieschke and Currie 2007). As a vertebrate model, zebrafish has revealed particular characteristics of various gene expression changes implicated in AD which cannot be observed in other animal models (Liao et al. 2012; Newman et al. 2012). A mutant zebrafish that lacks Psen1 (orthologue of PS1) has

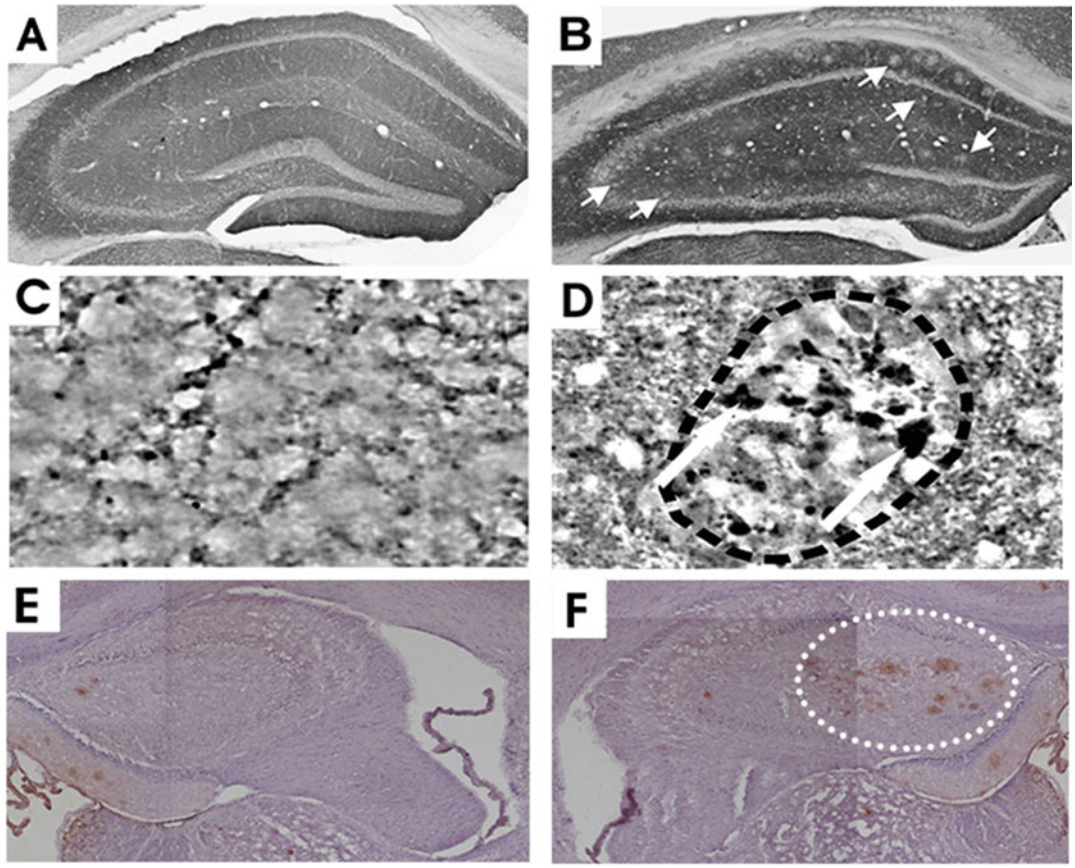

Fig. 4.2 Aβ plaques formed in the hippocampus of control and APP/PS1 transgenic mice (modified from Li et al. 2016). Arrows indicated that Aβ plaques in Panels (**b**), (**d**), and (**f**) compared to that of control mice in Panels (**a**), (**c**), and (**e**)

been developed to study the cognitive functions affected in AD. Analysis of histamine neurons in the *psen1*$^{-/-}$ zebrafish brains demonstrated that *psen1* in zebrafish is a regulator of histaminergic neuronal development (Sundvik et al. 2013). Inhibition of Psen2 protein translation in zebrafish has major effects on Notch signaling in comparison to Psen2$^{-/-}$ mice which show a minor phenotype (Herreman et al. 1999; Nornes et al. 2008, 2009). Furthermore, loss of Psen2 expression affects the production of interneurons in dorsal longitudinal ascending (DoLA) of the developing spinal cord in zebrafish larvae (Nornes et al. 2009). Translational blockers of morpholinos have also been employed to study the function of the APPa and APPb proteins (Orthologs of human APP) (Joshi et al. 2009). A previous study showed that only the full-length APP, but not the truncated one, can rescue the defects of neuronal activity, suggesting requirement of both extracellular and intracellular domains of human APP for normal brain function (Song and Pimplikar 2012). These studies demonstrated that zebrafish embryos can be used to study different mutations of human APP to uncover the molecular pathological mechanisms of AD.

4 Current Small Molecules and Antibodies for Treatment of AD

Earlier neurochemical studies indicated dysfunction of the cholinergic neurons resulted in the decrease of choline acetyltransferase (ChAT) activity, choline absorption, and acetylcholine

release in AD patients. Based on this mechanism, there are four cholinesterase inhibitors (tacrine, donepezil, rivastigmine, or galantamine) were developed and clinically usable. Later on, an N-methyl-D-aspartate (NMDA) receptor antagonist (Memantine) was developed for the treatment of AD. In addition, some small molecules and growth factors to inhibit Aβ deposit-induced neurodegeneration and activate endogenous neural regeneration are in the ongoing clinical trials (Coric et al. 2012; Dodel et al. 2013; Kang et al. 2009; Nygaard and Strittmatter 2009). The administration of a novel small molecule, stemazole, increased the memory and cognitive functions, and reduced the amount of Aβ1–40 aggregates in the hippocampus of rat AD model (Han et al. 2011). Some monoclonal antibodies and vaccines were also developed against Aβ deposit. In addition, inhibitors of γ- or β-secretase were developed on clinical trials to reduce the formation of Aβ plaques. Some short peptides corresponding to the active regions of BDNF were also found to increase neurogenesis in ex vivo hippocampal cultures from mouse embryonic day 18 (E18) fetus, indicating BDNF-related peptides could regulate BDNF signaling to increase neurogenesis in vivo (Blanchard et al. 2010; Cardenas-Aguayo Mdel et al. 2013). Currently there are more than one hundred small molecules and immunotherapies in the clinical trials of AD, of which 25 trials are in phase I, 52 agents are in phase II and 28 agents in phase III (Cummings et al. 2014, 2017). A Phase III clinical trial using monoclonal antibodies was shown to improve Aβ deposits but failed to improve cognitive dysfunctions of the patients (Gilman et al. 2005; Rinne et al. 2010). Since the majority of the Aβ-based treatments show no efficacy or have some toxicity in clinical trials, new therapeutic approaches are needed for the treatment of AD (Mullane and Williams 2013). With the identification of molecular targets of nicotinamide phosphoribosyltransferase (NAMPT), nuclear erythroid 2-related factor (Nrf2) and signaling pathways of Wnt/β-catenin pathway in neurogenic processes, more therapeutic agents have been developed and tested for the treatment of AD (Herrera-Arozamena et al. 2016).

5 Human Stem Cell–Based Model of AD

To develop effective therapeutics for AD, AD pathogenesis is needed to be deeply explored and understood. Because of the treatment failure in clinical trials with therapeutic drugs for AD, human disease models are needed to overcome the disadvantages of animal models. Recently iPSC-derived neural cells from patients with genetic mutations have been used to recapitulate key aspects of neural death in AD (Essayan-Perez et al. 2019). Once the iPS cells differentiate to the neural stem cells or neurons, they will lose the typical markers of iPS cells such as OCT4, NANOG, TRA-1-60 (Han et al. 2015). One study generated iPSC-derived neurons and astrocytes from familial and sporadic AD patients. It was found that Aβ oligomers are accumulated in these neural cells from familial patients with APP-E693Δ mutation and sporadic AD. The accumulated Aβ oligomers were shown to lead to oxidative stress and damage to endoplasmic reticulum (ER) of neural cells (Essayan-Perez et al. 2019).

Another study generated iPS cell lines from familial AD patients carrying APP mutation (V717I) and differentiated the iPS cells to forebrain neurons. They found that APP expression and levels of Aβ were increased dramatically in these iPS cell–derived neurons. They also found that the APPV717I mutation leads to increased levels of both APPsβ and Aβ by increased β-secretase cleavage of APP, affecting the initial cleavage site of γ-secretase to result in an increased Aβ42 and Aβ38. This study showed the previously unrecognized effects of the most common familial APP mutation V717I for studying the pathogenesis of AD (Muratore et al. 2014). To establish an in vitro cell model for studying the pathological mechanism of sporadic AD, a study investigated the iPS cell–derived neurons from FAD patients with PS1 mutations and SAD patients and compared the iPS cell–derived neurons with those of control individuals. The increased phosphorylation of TAU protein at all investigated phosphorylation sites was observed in neurons from both FAD and SAD

patients. Even though neurons derived from FAD and SAD patients showed higher levels of extracellular Aβ1–40 and Aβ1–42, only FAD patients have significantly increased Aβ1–42/Aβ1–40 ratios. Furthermore, the increased levels of active glycogen synthase kinase 3 β, a physiological kinase of TAU and elevated sensitivity to oxidative stress were detected in neuron-derived iPSCs from both FAD and SAD patients. This study indicated that in vitro iPS cells are suitable for modelling both FAD and SAD and are used for developing new therapeutics for AD. (Ochalek et al. 2017). One limitation to use iPS cells to model AD is that iPS cell–derived neurons lack the maturity and aging signatures of AD patients. Since iPSCs reprogrammed from aging somatic cells lose some aging profiles, iPSC-derived neurons from AD patients display a fetal and immature phenotype (Studer et al. 2015). Therefore, the current iPS cell models for AD need to be eventually improved to recapitulate the aging signatures of degenerated neurons in AD patients.

6 Stem Cell Therapy for AD

Advance in stem cell technology has provided a perspective approach for the treatment of incurable neurodegenerative diseases including AD. This is to use the transplanted stem cells to replace the degenerated or injured neural cells and activate the endogenous neurogenesis (Taupin 2006). The available stem cell sources include human embryonic stem cells (hESC), neural stem cells (NSC), mesenchymal stem cells (MSC) from bone marrow or other tissues such as the umbilical cord or olfactory ensheathing cells (OEC), induced pluripotent stem cells (iPSC), and the directly induced neurons (iN) from somatic cells (Wang et al. 2015).

6.1 Fetal Neural Stem Cell (NSC)

NSCs usually exist in the mammalian brain and have self-renewal and neural differentiation potentials. They are able to differentiate to different neurons and glial cells in vitro and in vivo after transplantation into the animal models or patients (Bond et al. 2015). Animal studies showed that NSCs from mouse embryos at postnatal day 14 were able to improve memory deficits after being transplanted to the hippocampus of the mouse AD model. The grafted NSCs in the hippocampi of the mouse brain were found to differentiate into neurons and astrocytes and survive for at least 5 months (Yamasaki et al. 2007). Electrophysiological studies provided evidence that the transplanted NSCs were able to significantly improve synaptic connections and increase the neuronal number of host mouse brain, indicating NSCs played some therapeutic roles in transgenic AD mice. After being transplanted to the triple-transgenic AD mouse model, NSCs improved the memory functions of the mice although the pathological aggregated plaques were not changed in this AD model (Blurton-Jones et al. 2009). NSCs were bilaterally transplanted to hippocampal regions of APP/PS1 transgenic mice and showed improvement of their function of spatial learning and memory. It was reported that NSCs did proliferate, migrate, and differentiate into neuronal cell types in vivo, but Aβ deposits in the brains of these mice could not be reduced, suggesting the pathological process is hardly reversed by the transplanted cells (Zhang et al. 2014).

Human neural stem cells (hNSCs) have also been widely studied for the treatment of AD. The therapeutic effects of hNSC were evidenced by a study which showed that transplantation of hNSCs were able to promote cognition in 3xTg-AD mice. The transplanted hNSCs can migrate and differentiate into neurons and astrocytes and increase synaptic connections with host brain neuronal cells of the AD mouse model (Ager et al. 2015). A recent study reported that bilaterally transplanted hNSCs into the hippocampus of an APP/PS1 double transgenic mouse model of AD improved the recognition and memory deficits of these mice. The transplanted hNSCs migrated dispersedly in broad brain regions and differentiated into the neurons and astrocytes (Fig. 4.3a, b). The synaptic and nerve fibers of the frontal cortex and hippocampus were found to be significantly increased in the hNSC-treated AD

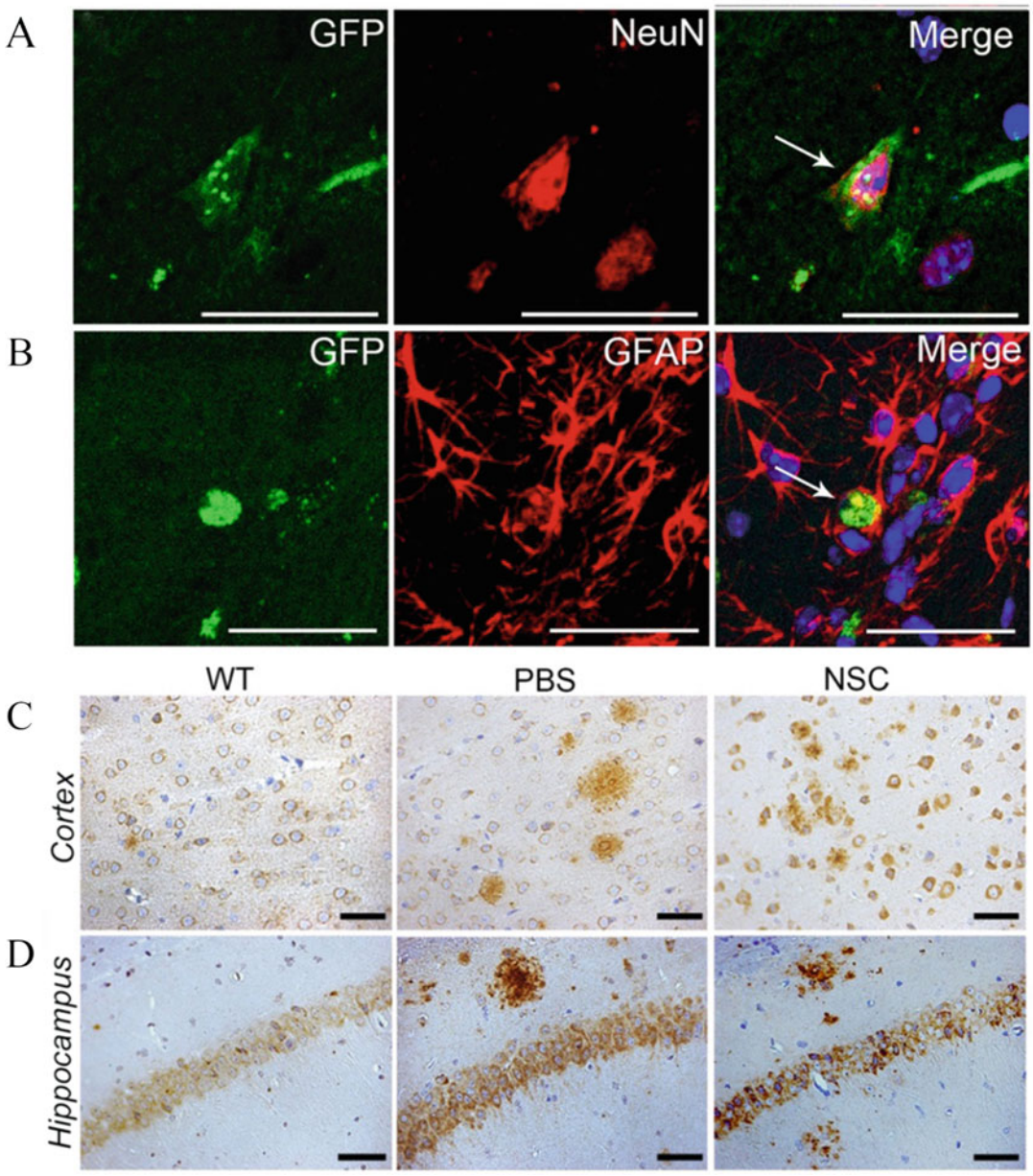

Fig. 4.3 Human neural stem cell transplantation in transgenic mice model with AD. (**a**) GFP-labeled hNSCs differentiated into mature neurons. (**b**) GFP-labeled hNSCs differentiated into GFAP-astrocytes scale bar: 50 μm. (**c**, **d**) Aβ plaques were decreased in frontal cortex (**c**) and hippocampus (**d**) of the mouse brains with transplanted hNSC compared to that in wild-type mice and PBS-transplanted APP/PS1 mice. Scale bar: 20 μm (modified from the study by Li et al. 2016)

mice, indicating the functional connectivity between transplanted hNSC and host brains of AD mice. Importantly, the Aβ plaques were found to be reduced in frontal cortex and hippocampus of the mouse brains with transplanted hNSC compared to that in wild-type and PBS-transplanted APP/PS1 mice (Fig. 4.3c, d) (Li et al. 2016).

To increase the survival and neural differentiation of transplanted cells in vivo, some genetic approaches were ever used to modify hNSCs to express cellular nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). After transplantation to AD animal models, the engineered NSCs expressing BDNF and NGF were shown to increase the function of spatial learning and memory in the APP/PS1 mice. These cells were also found to express NGF and improve the learning and memory function in chemically induced AD rats (Fan et al. 2014; Lee et al. 2012). Since cholinergic neurons are mainly affected to lead to decreased activity of choline acetyltransferase (ChAT), hNSCs were genetically modified to express ChAT gene to increase the release of ChAT for the restoration of cognitive function in AD. It was found that transplantation of hNSC expressing ChAT to AD rats, the learning and memory function of the rats were improved with the elevation of ACh levels in cerebrospinal fluid of the rats, suggesting the engineered hNSCs functioned in vivo (Park et al. 2012a, b). Another type of hNSCs is human olfactory bulb neural stem cell/olfactory ensheathing cell (OEC) which is isolated from the olfactory bulb tissue of adults or fetus. OECs were able to secrete neurotrophic factor to increase endogenous neurogenesis and the survival of the transplanted cells. Some studies indicated that OECs have been co-transplanted with NSC or other cells to repair brain and spinal cord injuries (Huang et al. 2012; Sun et al. 2013; Wang et al. 2010). The expression of choline acetyltransferase was significantly increased in co-transplanted animals than that in animal transplanted with olfactory ensheathing cells or neural progenitor cells alone to improve cognitive dysfunction in the rat model (Srivastava et al. 2009). The transplantation of neural progenitor cells (NPCs) combined with OECs to hippocampal regions promoted better recovery of learning and memory of animals lesioned with kainic acids (Srivastava et al. 2009). The human olfactory bulb-derived neural stem cells (OBNSCs) have also been engineered to express human nerve growth factor (NGF) by lentivirus-mediated transduction, and these cells were shown to restore cognitive deficit in the rat AD model after being transplanted into the hippocampus of AD rats induced by ibotenic acid. Importantly OBNSCs-hNGF cells were able to be differentiated to mature neurons and glial cells, including oligodendrocytes and astrocytes, and alleviate the memory and learning deficits of the AD rats (Marei et al. 2014).

6.2 Embryonic Stem Cell–Derived NSCs

Because of the limited source of fetal brain–derived NSC cells, pluripotent embryonic stem cells (ESCs) have been differentiated to NSCs for transplantation treatment of AD. To track the transplanted cells, EGFP-expressing mouse ES cells were differentiated into Nestin-positive NSCs which were then transplanted into the aggregated beta-amyloid (Aβ) peptide induced rats. It was found that these grafted NSCs into the hippocampus of the rats significantly improved the memory dysfunction of the A-β-injured rats 16 weeks after transplantation (Tang et al. 2008). A study ever compared the therapeutic effects of ESC-derived neuronal precursor cells (ESC-NPCs) and the Shh-primed ESC-NPCs (ESC-PNPCs) for the treatment of rat AD models. The Shh-Primed NPCs were induced from ESCs by adding Shh to the medium for neural induction before transplantation. As a result, Shh-Primed NPCs were found to differentiate better into cholinergic neurons. After transplantation, both ESC-NPCs and ESC-PNPCs improved the memory deficits of AD rats. This suggested that the transplantation of mouse ESC-NPCs and/or ESC-PNPCs (commitment to cholinergic cells) can promote behavioral recovery in the rat model of AD (Moghadam et al. 2009). The mouse and human ESCs have also been differentiated into mature basal forebrain cholinergic neurons (BFCNs) for transplantation therapy. After transplantation into the basal forebrain of AD mice, both mouse and human ESC-derived BFCN progenitors were able to differentiate into mature cholinergic neurons in vivo and improved the learning and memory functions

of AD mice (Yue et al. 2015). These studies proved that both mouse and human ESCs-derived NSCs are potential therapeutic cells for the treatment of AD.

6.3 Mesenchymal Stem Cells

Mesenchymal stem cells (MSC) are derived from different tissues such as adult bone marrow (BM-MSC), umbilical cord (UC-MSC), and umbilical cord blood (UCB-MNC). Human UC-MSC was ever induced to differentiate into neuron-like cells (HUMSC-NCs) under tricyclodecan-9-yl-xanthogenate (D609), and transplantation of HUMSC-NCs into an APP/PS1 transgenic AD mouse model showed improved cognitive function, increased synapsin I level, and reduced Aβ deposition in the brains of APP/PS1 mice (Bae et al. 2013; Yang et al. 2013b). Lee et al. reported that they injected amyloid-beta into the hippocampus of C57BL/6 mice to induce the AD models, and transplanted human BM-MSCs significantly reduced Aβ deposits in the brains of these AD mice. They also transpanted human BM-MSC into the hippocampus of APP/PS1 transgenic mice and found that grafted human BM-MSC reduced Aβ deposits, improved defective function of the microglia, and decreased the responses of inflammation. BM-MSC-treated APP/PS1 mice were also found to have improved cognitive function and decreased tau hyperphosphorylation. This study suggested that the transplantation of BM-MSCs was able to reduce the Aβ deposition probably by the mechanism of the microglial activation (Lee et al. 2009, 2010). Another study found that the deposition of cerebral Aβ was significantly decreased in young AD mice and showed that pre-synaptic proteins of dynamin 1 and synapsin 1 which are typically decreased in the brains of AD patients were upregulated in the brains of AD mice treated with BM-MSCs (Bae et al. 2013). A recent study suggested that the central nervous system inflammation plays an important role in AD pathogenesis (Rubio-Perez and Morillas-Ruiz 2012). Bone marrow–derived MSCs over-expressing vascular endothelial growth factor (VEGF) were transplanted into transgenic mice and induced neovascularization and improved memory function of the mice (Garcia et al. 2014). In addition, transplantation of human adipose-derived stem cells reduced memory and cognitive deficits of the AD mice (Chang et al. 2014). Interestingly, transplantation of T regulatory cells (Tregs) in combination with UC-MSCs to the APP/PS1 double-transgenic AD mice has been shown to reduce the deposition of the Aβ plaque and to improve the impaired cognitive dysfunction. Furthermore, the soluble Aβ was found to be increased and systemic inflammational factors were decreased, indicating that co-transplantation of T regulatory cells (Tregs) and MSCs plays some synergistic roles for the treatment of AD (Yang et al. 2013a).

6.4 Induced Pluripotent Stem Cells (iPS Cells)

Although transgenic/knockout animal models provided important applications on the pathological and therapeutic mechanisms of AD, these models cannot fully represent the human phenotype and pathology of AD by genetically editing the specific mutations in the AD genes (Duff and Suleman 2004). Recent advance in somatic cell reprogramming has generated iPS cells by over-expressing 2–4 transcription factor genes of OCT4, SOX2, c-MYC, and KLF4 in skin fibroblast cells, blood cells, and urine cells. The advantage of iPS cells is that these cells are derived from patients themselves and can be induced to differentiate into any type of cells, including neurons and glia. Thus iPS cells can be transplanted autologously without immune rejections and ethical concerns of heterogeneous stem cell transplantation and will have great value for cell therapy of neurodegenerative diseases such as AD (Park et al. 2008; Takahashi et al. 2007; Yu et al. 2009; Zhou et al. 2012).

Several iPS cell lines have been derived from AD patients with mutations in *PS1* (A246E), *PS2* (N141I) and APP genes and have been differentiated to neurons and cholinergic neurons to model the pathological changes

of AD. By studying these iPS cell–derived neurons, the secretion of Aβ-42 could be modified by some γ-secretase inhibitors. This indicated that AD patient–derived neurons can be potentially used as human cell model and to screen for therapeutic drugs for AD (Yagi et al. 2011). iPS cell–derived neurons with a genetic duplication of the *APP* gene were seen to have significantly increased Aβ deposits, phosphorylated-Tau and active glycogen synthase kinase-3β (aGSK-3β). The treatment of the iPS cell-derived neurons by β-secretase inhibitors significantly reduced the level of phosphorylated Tau and aGSK-3β (Israel et al. 2012). In another study human iPSCs-derived macrophages were engineered to express neprilysin-2, the Aβ-degrading protease, and were able to therapeutically reduce Aβ levels after being transplanted to a transgenic mouse model of AD (Takamatsu et al. 2014).

Since the retroviral or lentiviral-induced iPS cells may have integration of exogenous genes of OCT4, SOX2, c-MYC and KLF4 and are not suitable for clinical therapy, transgene-free iPS cells were derived from AD patients with PS1 mutation (A246E) using non-integrating episomal vectors. Neurons that were derived from mutant iPSC lines were shown to express PS1-A246E mutations and to have amyloid-β deposits as shown in brain of AD patients. These iPS cells that harbor *PS1* gene mutation could be utilized as human models to study AD pathogenesis and to screen therapeutic drugs for AD (Machairaki et al. 2014). Recently protein-based reprogramming can generate iPS cells more suitable for clinical therapy. It was reported that protein-based iPSCs were able to be differentiated into glial cells and decreased plaque depositions in the 5XFAD transgenic AD mouse model. The transplanted neurons derived from these iPSCs were found to mitigate the cognitive dysfunction in these mice (Cha et al. 2017; Han et al. 2019).

However, the iPS cell–derived neurons from AD patients may harbor genetic mutations and cannot be transplanted directly to AD patients as a therapy. To resolve this issue, several approaches, including CRISPR-cas9 technology, have been developed to correct the mutant iPS cells by homologous recombination as demonstrated in other studies. Then, these mutation-corrected neurons could be transplanted to patients for the treatment of AD (Garate et al. 2013; Hockemeyer et al. 2011; Moreno et al. 2018; Ortiz-Virumbrales et al. 2017).

6.5 The Molecular Mechanisms of Stem Cell Transplantation

Stem cell transplantation is able to improve memory and cognitive function in the animal models of AD and some clinical trials on AD patients. However, its therapeutic mechanism is not quite known. The possible mechanisms could include direct cell replacement of the injured neural cells, released neurotrophic factors and neuroprotective factors to increase cell survival, activation of endogenous stem cells, and modulate the host immune reactions. Stem cell-secreted Brain-derived neurotrophic factors (BDNF), glia-derived neurotrophic factor (GDNF), and nerve growth factor (NGF) are the major factors playing regulatory functions in synaptic plasticity. In addition, transplanted stem cells can carry and deliver therapeutic proteins to the degenerated regions of brain to decrease Aβ deposits. Neural stem cells can be engineered to express many neurotrophins, including BDNF and NGF (Blurton-Jones et al. 2009; Wu et al. 2016). Rats with traumatic brain injury (TBI) were intravenously transplanted with hMSCs and were evaluated at different times after transplantation. The extracts from the entire traumatized cerebral hemispheres with grafts 24 h after TBI showed significantly increased expression of nerve growth factor (NGF), brain-derived neurotrophic factor, (BDNF), and neurotrophin-3 (NT-3) (Kim et al. 2010). Transplanted NSCs can also induce endogenous neurogenesis of the hosts, and improve functional outcome. In a study for the treatment of distal middle cerebral artery occlusion (MCAO) in rats, the transplanted NSCs derived from human embryonic stem cells can increase neurogenesis identified by the expression of doublecortin (Dcx) in subventricular zone (SVZ), but neurogenesis did not occur in

contralateral SVZ or dentate gyrus zone (SGZ) of the rat brains 60 days after transplantation (Jin et al. 2011). A recent study even demonstrated that adipose-derived MSC transplanted into the hippocampus of APP/PS1 double transgenic AD mice significantly increased the number of BrdU/DCX-labeled cells in the dentate gyrus of the hippocampus, suggesting that transplantation of MSC improves memory and cognitive functions by enhancing the neurogenesis of the APP/PS1 transgenic AD mice (Yan et al. 2014).

In a study that aims to reduce the Aβ deposit, the NSCs from postnatal mice were genetically modified to express metalloproteinase 9 (MMP9), a protease to degrade Aβ peptide aggregates and therefore to alleviate amyloid pathology. However, stem cell–delivered MMP9 was found to have no impact on Aβ plaques, suggesting that the delivering approach may need to be optimized for improving the efficacy of therapy from transplanted cells (Njie et al. 2012). To know how transplanted cells improve spatial memory, hNSC cells were injected into the cerebral lateral ventricles of APP-expressing transgenic mice at 13 months of age. It was found that Aβ production was reduced through an Akt/GSK3-β-signaling-mediated decrease in BACE1, and expression of inflammatory mediators was decreased through deactivation of microglia (Lee et al. 2015).

7 Clinical Studies of Stem Cell Transplantation

After the safety and efficacy of stem cell transplantation has been obtained through animal models with AD, clinical trials were approved to transplant stem cells to AD patients. One clinical study injected human umbilical cord blood-derived mesenchymal stem cells to patients and found that the memory function of the patients was improved (Kang et al. 2016). Another clinical trial enrolled 9 patients with Mini-Mental State Examination scoring (MMSE) between 10 and 24 (mild-moderate AD dementia) and evaluated the safety of allogeneic human umbilical cord blood-derived MSCs. Before transplantation, the positron emission tomography confirmed Aβ plaques in brains of all the patients. Three patients received low-dose cell transplantation (3×10^6 cells) while 6 patients were transplanted with high dose of cells (6×10^6 cells) bilaterally into the hippocampus and precuneus. At 3 and 24 months after transplantation, no patient showed any serious adverse effect resulting from either the surgical procedure or transplanted MSCs. However, the cell transplantation did not improve cognitive function in a 2-year follow-up observation. Furthermore, no pathological changes were improved in any of the patients (Kim et al. 2015). Other clinical studies used different transplantation routes, cell dosage, and objective biomarkers such as positron emission tomography to evaluate the therapeutic efficacy of transplanted cells in different clinical stages of AD patients. But no reliable results were obtained in these clinical trials using different stem cells for the treatment of AD. It is believed that advances in induced pluripotent stem cell (iPSC) technology will accelerate the development of stem cell–based therapeutic approaches for AD.

8 Conclusions

Since there is no effective treatment for AD, stem cell transplantation holds great potential as they can differentiate into neurons and glia to replace damaged neural cells or release cellular cytokines to activate the endogenous neurogenesis. By now, few trials showed positive results, suggesting there are many aspects related to the stem cell therapy are underway. Thus, not only do we need to better understand the mechanisms underlining the AD pathology that regulates the survival, proliferation, migration, differentiation, and function of transplanted stem cells, but also we should translate the results of animal studies to clinical trials on AD patients. Some stem cell sources, such as BM-MSC, UC-MSC, and UCB-MSC, are easily obtainable; however, their neural differentiation potential is limited and can be immunely rejected as they are allogeneic. Human ESCs and fetal NSCs have some ethical and immune rejection issues which restrict their clinical application for AD

patients. The newly developed iPS cells can overcome the clinical limitations of other stem cell sources, indicating that patient-specific iPS cells will have invaluable perspective for treatment of AD. Before iPS cells move forward to AD patients, some concerns and procedures, such as correction of mutations, neural induction and differentiation, neural cell purification, as well as long-term survival of iPS cell-derived neural cells after transplantation, need to be addressed, optimized, and standardized in order to make clinical GMP-grade iPS cells suitable for AD patients.

Acknowledgement This work was supported by National Natural Science Foundation of China (NSFC 81571241), Department of Science and technology of Shandong Province, China (2017GSF18104), and Basic Research fund from Natural Science Foundation of Shandong Province, China (ZR2019ZD39). We also thank Qingfa Chen, Chuanfei Wei, and Wei Wang in my lab for their technically editing the references and making figures of the manuscript.

Disclosure The authors have no conflict of interest in this work.

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Cell Replacement Therapy for Huntington's Disease

5

Ruth Monk and Bronwen Connor

1 An Introduction to Huntington's Disease

Huntington's disease (HD) is the most common inherited neurodegenerative disorder, affecting between 1 in 10,000 and 1 in 25,000 people worldwide (Dayalu and Albin 2015). HD is caused by a single autosomal dominant mutation, the effects of which lead to a highly debilitating and progressive decline of motor, cognitive, and mental functioning (Dayalu and Albin 2015; The Huntington's Disease Collaborative Research Group 1993). In 1993, the revolutionary discovery of the causative mutation of HD occurred with the identification of the IT15 'Huntington's disease gene' on chromosome 4 by the Huntington's Gene Collaborative Research Group (The Huntington's Disease Collaborative Research Group 1993). Although the identification of this gene was expected to lead to the rapid discovery of a cure for HD, 24 years of research has not yet been able to identify a way of reducing or preventing the progression of HD in individuals affected by the disease.

HD is caused by an expansion of the trinucleotide CAG repeat in the HD gene, resulting in the encoded ubiquitously expressed Huntingtin (HTT) protein containing excessive polyglutamine stretches near the N-terminus (The Huntington's Disease Collaborative Research Group 1993). In unaffected individuals, the HTT protein contains between 6 and 35 CAG repeats. Conversely, when the number of CAG repeats exceeds 35, a mutant Huntingtin protein (mHTT) is produced, giving rise to the symptoms of HD (The Huntington's Disease Collaborative Research Group 1993). Individuals with 40 or more repeats are guaranteed to develop HD, although incomplete penetrance of the mutation may cause some individuals with between 36 and 39 repeats to carry with mutation without experiencing symptoms of the disease (Dayalu and Albin 2015). Furthermore, CAG expansions containing between 27 and 35 repeats are classed as intermediate lengths which, with a few observed exceptions, are not causative of HD (Ha and Jankovic 2011). Individuals carrying these intermediate repeat lengths without experiencing HD are termed asymptomatic carriers; however, genetic anticipation and instability of the CAG repeat length still put these individuals at risk of transmitting the disease (Ha and Jankovic 2011).

In the majority of cases, HD symptom onset occurs between 35 and 50 years of age. Large family studies have exposed an inverse relationship between the length of the expanded CAG repeat and the age of onset and severity of HD (Zuccato et al. 2010). However, there are many exceptions to this inverse relationship, with between 30% and 50% of variation in the age of

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F. Han, P. Lu (eds.), *Stem Cell-based Therapy for Neurodegenerative Diseases*, Advances in Experimental Medicine and Biology 1266, https://doi.org/10.1007/978-981-15-4370-8_5

symptom onset being accounted for by non-length genetic differences, such as PCG1α and Δ2642 glutamic acid polymorphisms, as well as environmental factors (Landles and Bates 2004; Ross and Tabrizi 2011). Consequently, the clinical presentation of HD symptoms can be relatively variable in the timing of onset, order in which symptoms appear, and individual severity. To address this variability, the symptoms of HD are frequently grouped into a triad of dysfunctions, consisting of progressive motor disorder, cognitive impairments, and psychiatric disturbances (Dayalu and Albin 2015; Zuccato et al. 2010; Landles and Bates 2004).

HD is characterised by the preferential degeneration of GABAergic medium spiny neurons (MSNs) in the striatum. Up to 95% of MSNs may be lost in HD, with MSNs containing encephalin being especially vulnerable to such degeneration (Cepeda et al. 2007). In individuals with HD, the Huntingtin protein is affected by a toxic gain-of-function mutation and subsequently accumulates in neurons (Cepeda et al. 2007; Juopperi et al. 2012; Jeon et al. 2012). In addition to the striatum, cell death may also occur in the cerebral cortex, white matter, thalamus, and hypothalamus, albeit to a lesser extent (Ross and Tabrizi 2011). Some of the symptoms of cognitive dysfunction in HD, such as impairments in executive functioning, have been linked to morphological changes in their corresponding brain regions; however, these imaging studies have been unable to determine if observed changes are indicative of cell dysfunction or death (Zuccato et al. 2010). Ultimately, the preferential loss of MSNs observed in HD is largely disproportionate to the presence of HTT, which is ubiquitously expressed throughout the brain (Dayalu and Albin 2015). These discrepancies emphasis the need to elucidate why MSNs preferentially degenerate and the specific mechanisms through which mHTT results in the observed pattern of regionally selective neurodegeneration.

One of the most significant obstacles in finding a treatment for HD is the limited knowledge of the precise mechanisms in which mHTT leads to the characteristic neuropathology and symptoms of HD. Despite a simple genetic cause, the specific mechanisms that lead to the pathogenic effects and highly debilitating symptoms of HD are undoubtedly complex. As summarised in Fig. 5.1, mHTT affects a wide range of molecular pathways and functions, which are thought to interact and culminate in MSN loss and HD symptomatology. Among these pathways, particular interest has been given to the roles of reduced brain-derived neurotrophic factor (BDNF) (Zuccato et al. 2003, 2008; Gauthier et al. 2004), abnormal protein processing (Sakahira et al. 2002), excitotoxicity (Sun et al. 2001; Cepeda et al. 2008), and mitochondrial dysfunction (Tang et al. 2005; Guo et al. 2013; Weydt et al. 2009) as key contributors to HD neuropathology. Ultimately, it is likely that HD results from complex interactions across many similar and distinct molecular pathways, as well as being influenced by genetic and phenotypic factors.

2 Cell Replacement Therapy for Huntington's Disease

Despite identifying the causative mutation 24 years ago, there are currently no disease-modifying treatments which are able to prevent HD progression or provide a cure for those affected by the disease. While reproductive technologies and genetic pre-implantation diagnosis can be utilised to prevent the transmission of the causative mutation of HD to the children of those affected, there are no preventative treatments available for those born with the mutation. Therefore, most current treatments for HD are centred on alleviating the burden of symptoms in individuals with HD.

A potential approach for the treatment of HD is the development of cell-based therapies. The goal of cell-based therapies is to both restore neuronal circuitry and function by replacing lost neurons and to provide neurotropic support to prevent further degeneration. In order to successfully restore basal ganglia functioning in HD, cell-based therapies would need to reconstitute the complex signaling network disrupted by extensive MSN degeneration. This chapter will

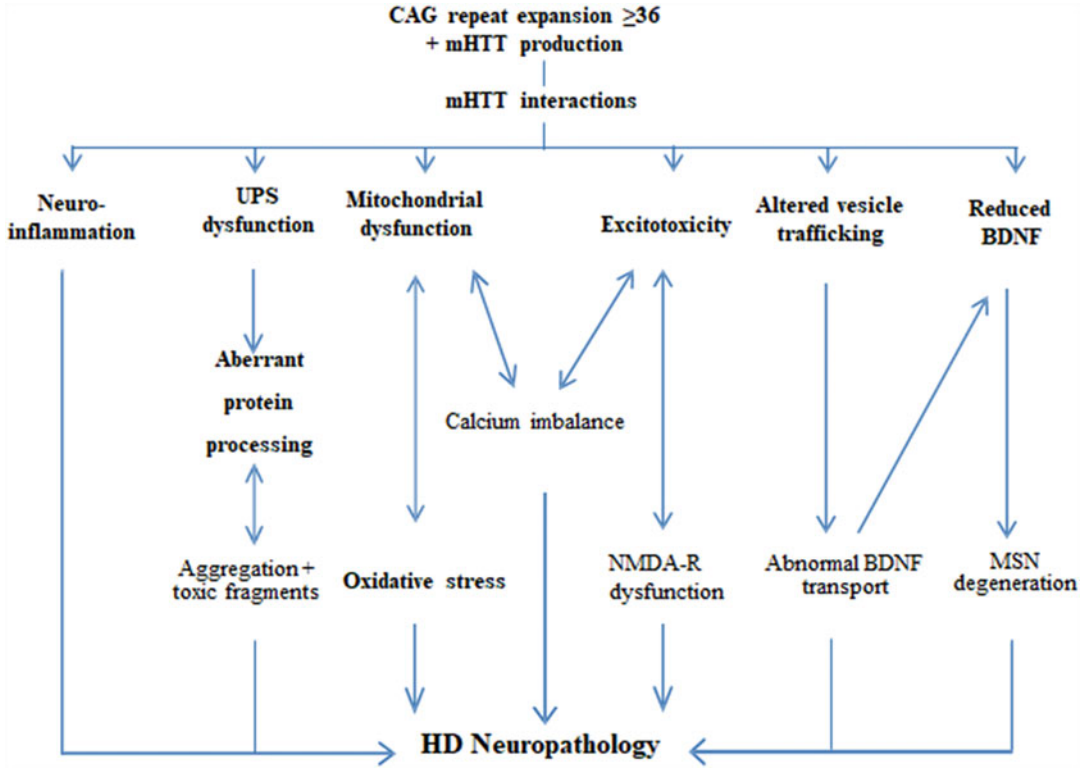

Fig. 5.1 Proposed mechanisms of mHTT-induced neuropathogenesis of HD. The presence of a ≥36 CAG repeat expansion results in the production of mHTT, which undergoes various interactions resulting in the dysfunction or abnormal activity across a range of inter-related pathogenic processes with varying contributions to MSN degeneration and HD pathology

discuss the potential use of foetal tissue grafts, pluripotent stem cells, neural stem cells, and somatic cell reprogramming to develop cell-based therapies for treating HD.

2.1 Foetal Tissue Grafts for Huntington's Disease

Traditionally, grafts of embryonic or foetal striatal tissue have been used in pre-clinical transplantation studies utilising rodents and non-human primate models of HD (Kendall et al. 1998; Dunnett et al. 2000; Deckel et al. 1986). Grafted foetal tissue is able to survive, successfully differentiate into striatal neurons, and integrate with the host networks. As well as demonstrating successful neuro-anatomic integration of grafted tissue, pre-clinical studies have provided proof-of-principle that transplanted foetal tissue can improve cognitive and motor symptoms and restore fine motor movement (Kendall et al. 1998; Deckel et al. 1986; Isacson et al. 1986; Pritzel et al. 1986; Sirinathsinghji et al. 1988; Clarke et al. 1988a, b; Dunnett et al. 1988a, b; Palfi et al. 1998; Nakao and Itakura 2000; Freeman et al. 2000a; Klein et al. 2013).

The promising results from animal studies prompted a rapid translation to humans, with several clinical trials conducted to assess the potential for foetal tissue grafts to treat patients with HD (Bachoud-Levi et al. 2000; Freeman et al. 2000b; Hauser et al. 2002; Rosser et al. 2002; Gaura et al. 2004; Furtado et al. 2005; Bachoud-Lévi et al. 2006; Farrington et al. 2006; Krystkowiak et al. 2007; Gallina et al. 2008; Reuter et al. 2008; Barker et al. 2013).

Despite the vast amount of clinical data generated by these trials, it is difficult to compare between studies due to inherent differences in study design, exclusion of appropriate controls, potential physician bias, and variation in the methods of assessing clinical and motor functioning (Benraiss and Goldman 2011). Overall, these trials produced modest results with some improvements in motor and cognitive functioning; however, these improvements could not be preserved long term. The loss of foetal graft tissue over time was suggested to be a result of insufficient neurotrophic support and sub-optimal methods of graft preparation contributing to reduced cell viability and a low survival rate in vivo (Chen et al. 2014). While single-cell suspensions of foetal striatal cells demonstrate increased survival in HD rodents, their use is fraught with ethical issues, limited availability, and population heterogeneity (Cisbani et al. 2014). Ultimately, the transient symptom improvement exhibited by HD patients who received foetal tissue grafts was unable to prevent the progression of the disease or significantly improve patient survival.

The use of foetal cell therapy in HD is severely hindered by a host of ethical, technical, and safety issues, which must be taken into consideration. The obtainment of foetal tissue from aborted foetuses raises serious ethical concerns, and the limited availability of such tissue calls into question the practicality of foetal cell grafts as a common treatment for HD (Chen et al. 2014). Adding another level of complication, maintaining the viability of foetal tissue during its preparation can be extremely challenging due to difficulties retaining an adequate blood supply and avoiding contamination (Isacson and Breakefield 1997). Furthermore, the scarcity of human foetal tissue and controversy surrounding its use have resulted in variability between studies as they differ in the age and source of the foetal tissue used for grafting, adding an additional complication when trying to compare results from various clinical trials (Benraiss and Goldman 2011). Another source of variation arises from differences in tissue preparation and immunosuppression between studies. Although multiple long-term studies evaluating clinical safety and efficacy of foetal grafts into HD patients reported no tumour formation or adverse effects from the grafts at approximately 10 years post-transplantation (Barker et al. 2013; Cisbani et al. 2014), other studies have generated conflicting results, with patients experiencing graft cell overgrowth, alloimmunisation, and rejection of the grafted tissue (Hauser et al. 2002; Krystkowiak et al. 2007). While pharmacological immunosuppressive treatment can prevent or reverse these reactions, ongoing immunosuppression is impractical and raises additional safety concerns (Krystkowiak et al. 2007; Benraiss and Goldman 2011). Consequently, there has been a high demand to identify alternative sources of cells for treating HD using cell-based therapies.

2.2 Human Neural Stem Cells

One option to provide an alternative source of cells for transplantation is the use of neural stem cells obtained from human foetal brain (hNSCs). While this still requires the use of tissue from aborted foetuses, the ability to expand and generate immortalised lines of hNSCs allows for a homogenous population of cells with significantly reduced variation and technical issues, as well as less donor tissue requirement. Ryu and colleagues demonstrated that transplantation of hNSCs into the 3-nitropropinoic acid (3-NP) lesion model of HD improved motor function impairment when the hNSCs were transplanted prior to lesioning, while transplantation after lesioning did not result in motor improvement (Ryu et al. 2004). Interestingly, the transplanted hNSCs exhibited endogenous BDNF secretion both before and after transplantation, suggesting that the observed motor improvement may result in part from neurotrophic support provided by the transplanted cells.

A similar study examined the effect of culturing and pre-differentiating hNSCs in the presence or absence of cillary-derived neurotropic factor (CNTF) prior to transplantation into the striatum of the quinolinic acid (QA) lesion model of HD (McBride et al. 2004). The hNSCs cultured in the

presence or absence of CNTF yielded similar survival and migration patterns following transplantation, as well as generating significant improvements in motor function after 8 weeks. However, the presence of CNTF was associated with significantly reduced striatal atrophy following transplantation, highlighting the beneficial effect of transplanting cells that provide additional neurotrophic support.

Subsequent studies examined the ability of hNSCs to migrate to the site of lesioning when injected into the ventricles or tail vein of QA lesioned rodents (Lee et al. 2005, 2006). These studies verified that hNSCs could successfully migrate into the striatum and improve motor functioning as in previous studies, providing less invasive methods of cell administration and increasing the clinical applicability of hNSC transplantation for translation into HD patients.

2.3 Pluripotent Stem Cell Therapy for Huntington's Disease

Human pluripotent stem cells (hPSCs) from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) have received a lot of attention for their potential as a cell-based therapy for neurodegenerative disorders. Unlike human foetal tissue grafts, hPSCs are expandable and are not sourced from aborted foetal tissue, providing a more obtainable and less controversial source of cells for cell transplantation studies.

Due to the highly proliferative nature of hPSCs, engraftment of these stem cells directly into the brain confers a substantial risk of tumourigenesis (Kulbatski 2010). In fact, transplantation of ESCs or iPSCs into immunocompromised mice is commonly used to confirm the pluripotency of transplanted cells upon teratoma formation (Takahashi et al. 2007; Takahashi and Yamanaka 2006; Ross et al. 2011). Consequently, stem cell transplantation studies differentiate hPSCs towards hNSCs in order to reduce the risk of tumourigenesis and increase lineage specificity following transplantation (Mattis and Svendsen 2015). Early studies demonstrated that hESC-derived NSCs transplanted into the QA lesion rodent model of HD survived and had beneficial effects on rescuing QA-induced motor function impairment (Joannides et al. 2007; Song et al. 2007; Vazey et al. 2010). However, the transplanted cells did not differentiate into region-specific neurons which expressed MSN markers (Joannides et al. 2007; Song et al. 2007; Vazey et al. 2010).

To address this limitation, multiple groups differentiated hESCs into lateral ganglionic eminence (LGE) progenitors or striatal precursor cells in order to increase lineage specificity and encourage differentiation into MSNs, albeit with a range of different protocols and a variety of MSN yields (Ma et al. 2012; Delli Carri et al. 2013; Aubry et al. 2008; Arber et al. 2015; Nicoleau et al. 2013). While multiple studies reported functional integration of transplanted cells and improved performance on motor function tests (Ma et al. 2012; Delli Carri et al. 2013), these results were not replicated by similar studies (Aubry et al. 2008; Arber et al. 2015). These disparities may be due in part to differences in the maturity and number of cells transplanted between studies, revealing a need to compromise between over-growth associated with higher numbers of transplanted cells and an absence of behavioural rescue associated with lower numbers of transplanted cells (Delli Carri et al. 2013; Arber et al. 2015; Nasonkin et al. 2009). Nonetheless, hPSCs remain a promising source for cell-based HD therapies, with hopes that optimised protocols and increased yields of MSNs will result in greater therapeutic benefits in rodent models and allow for the eventual translation of these therapies to patients with HD.

With the discovery of iPSC reprogramming technologies, multiple groups have turned to iPSCs as a source of hPSCs for use in cell replacement therapies for HD (Jeon et al. 2012; An et al. 2012). Importantly, the nature of iPSCs as patient-specific allows for autologous transplantation, which avoids the issues of immune rejection associated with hESCs and the requirement for immunosuppression following transplantation (Chen et al. 2014; Mattis and Svendsen 2015). However, the genetic nature of HD limits the use of patient-derived cells for cell replacement

approaches as the genetic component is retained in transplanted cells. In one study, hiPSC-derived NSCs generated from an HD patient with a CAG repeat length of 72 were transplanted into the QA lesion and the YAC128 transgenic rodent models of HD (Jeon et al. 2012). These NSCs differentiated into neurons expressing markers of MSNs (Jeon et al. 2012). At 12 weeks after the transplantation none of the cells which had been transplanted into either rodent model exhibited aggregates of human mHTT. However, by 33 weeks post-transplantation aggregates were detected in cells transplanted into the QA lesion model. Interestingly, the QA-lesioned rats demonstrated motor function improvement, even when transplanted with HD patient–derived hNSCs. Despite the beneficial results observed in this study, it is likely that the autologous transplantation of HD patient–derived cells carrying the HD mutation would eventually lead to cell death and persistence of the HD phenotype (Chen et al. 2014). Consequently, the genetic correction of the HD mutation has become an extremely attractive and rapidly advancing goal for cell replacement therapy with autologous cells. In fact, one study successfully corrected the mutation of an HD patient–derived iPSC line and generated hNSCs for transplantation into the R6/2 transgenic mouse model of HD (An et al. 2012). Although this study did not report any phenotypic effects, the transplanted cells survived and successfully differentiated into neurons expressing markers of MSNs. Ultimately this study provided the first evidence for the use of HD patient–derived cells which had been genetically corrected and transplanted into an animal model of HD.

Differentiating hPSCs towards a neuronal lineage prior to transplantation may reduce the risk of teratoma formation upon transplantation. However, the considerable risks of genetic mutagenesis and tumourigenesis remain as a result of the accumulation of chromosomal abnormalities in long-term cultures (Ross et al. 2011). In hESCs, the survival and differentiation of transplanted cells, as well as tumour formation, are dependent on the maturity of these cells (Aubry et al. 2008). Differentiating hESCs into hNSCs which were further along the MSN neuron development pathway prior to transplantation was inversely correlated with tumour formation for up to 6 weeks post-transplantation (Aubry et al. 2008). However, increasing the rate of differentiation of hPSCs into hNSCs prior to transplantation may also increase the risk of tumour formation if residual pluripotent stem cells which escaped differentiation persist in the differentiating cultures (Vazey et al. 2010; Brederlau et al. 2006). Indeed, substantial outgrowths of transplanted cells could be observed in animals 2 months after the transplantation of striatal progenitors derived from hESCs, calling into question whether the tumourigenic risk can be completely removed from hPSC-derived transplants (Aubry et al. 2008). Furthermore, iPSCs carry an additional risk of developing genetic abnormalities and insertional mutagenesis due to the use of oncogenic reprogramming factors and integrative methods of factor delivery (Takahashi et al. 2007; Takahashi and Yamanaka 2006). While there has not been any report of tumour formation following hPSC-derived hNSC transplantation therapies in rodents, it should be noted that the short lifespans of these models does not allow for sufficient monitoring of long-term effects (Benraiss and Goldman 2011). Thus, it is imperative that these methods are trialed on larger animals with similar lifespans to humans to examine long-term stability before the translation of these therapies to HD patients. Even following the successful evasion of tumourigenecity in such models, patients receiving hPSC-based cell replacement therapies would need to undergo frequent and robust monitoring to identify any early changes which could be indicative of genetic abnormalities or tumour formation (Chen et al. 2014).

2.4 Adult Neural Stem Cell Therapy for Huntington's Disease

An alternative method of obtaining neural progenitor cells for transplantation is the extraction of multipotent, self-renewing NSCs directly from

neurogenic regions of the adult brain (Vazey et al. 2006; Vazey and Connor 2010; Johann et al. 2007). In addition to reducing the ethical concerns arising from the use of foetal tissue or ESCs, the commitment of NSCs to a neuronal fate avoids the risk of teratoma formation resulting from the presence of undifferentiated PSCs when transplanted.

An initial study by Vazey and colleagues utilised NSCs from the subventricular zone (SVZ) of adult rats and demonstrated that allotransplantation into the striatum of the QA lesion model leads to a significant improvement in motor function (Vazey et al. 2006). While the majority of transplanted cells differentiated into astrocytes, ~35% of cells exhibited a mature neuron phenotype, of which ~15% of these expressed markers of MSNs. More so, priming these NSCs with lithium chloride prior to transplantation increased the yield of neurons expressing the MSN neuron marker DARPP32 to 34% at 12 weeks post-transplantation (Vazey and Connor 2010).

A subsequent study investigated the relationships between cell preparation prior to transplantation, the timing of cell transplantation, and the survival of transplanted cells in the QA lesion model of HD (Johann et al. 2007). This study demonstrated that the greatest survival of transplanted cells occurred when the transplantation took place only 2 days post-lesioning, and when cells were transplanted as neurospheres as opposed to dissociated cells. The reduced survival of cells when transplanted 1 or 2 weeks after lesioning may be a result of the activation of host glial cells and/or the increased expression of cytokines following QA lesioning (Johann et al. 2007; Gordon et al. 2009). As the striatal environment in brain of patients with HD is likely to differ to that of chemical lesion models of HD, it is important to consider how these lesion-dependent timings of transplantation would translate to patients with HD. Another caveat of this study is that the transplanted cells differentiated into astroglial cells expressing GFAP by 3 months post-transplantation, calling into question the ability of these transplants to replace the functional activity of the degenerated striatal cells and rescue the HD phenotype (Johann et al. 2007). This and other studies suggest that while the environmental cues required for MSN differentiation from NSCs may be present in the striatal region of the QA lesioned brain, non-neurogenic cues promoting the generation of glia are prominent in the lesioned brain (Gordon et al. 2007, 2009; Chen et al. 2007; Jones and Connor 2011, 2016). Further studies are needed to confirm whether these cues also exist in the brain in patients and non-lesion models of HD.

2.5 Induced Neural Stem Cell Therapy for Huntington's Disease

The recent advancements in somatic cell reprogramming and gene editing technologies are expected to revolutionise the field of cell replacement therapy for treating neurodegenerative disorders (Fig. 5.2). As with the generation of iPSCs, the recent discovery of direct reprogramming techniques would allow for the transplantation of autologous patient-specific cells in the absence of immunosuppression. Furthermore, directly reprogrammed cells have the additional advantage of avoiding pluripotency-associated tumourigenesis, as well as eliminating the requirement for oncogenic reprogramming factors.

To confirm that iNSCs could survive in vivo, Ring and colleagues generated iNSCs from mouse embryonic fibroblasts to transplant into the hippocampus or cortex of wild-type mouse pups (Ring et al. 2012). The iNSCs differentiated into neurons, astrocytes, and oligodendrocytes in vivo, and importantly, there was no evidence of tumour formation when iNSCs or wild-type brain-derived NSCs were transplanted. In contrast, more than 60% of mice which received iPSC-derived NSC transplants developed teratomas. As such, this study hypothesied that iNSCs represented a superior source of reprogrammed cells as they had little or no tumourigeneicity when transplanted. A similar study reinforced these findings by generating hNSCs from adult human fibroblasts (Mitchell

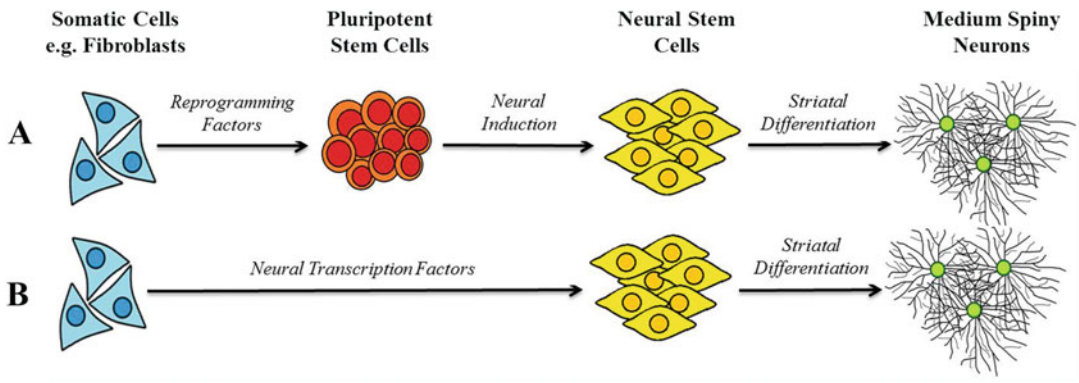

Fig. 5.2 Methods of MSN generation via somatic cell reprogramming. (**a**) MSNs can be generated from human somatic cells using induced pluripotent stem cell reprogramming or (**b**) direct reprogramming to a neural stem cell stage and subsequently MSNs. (**a**) Ectopic expression of the Yamanaka reprogramming factors converts fibroblasts into iPSCs which can undergo neural induction to form NSCs and subsequent differentiation into neurons expressing markers of MSNs, including DARPP32. (**b**) Fibroblasts can be reprogrammed using neural transcription factors to form iNSCs which can subsequently be differentiated into neurons expressing markers of MSNs

et al. 2014). Upon transplantation into immunodeficient mice, no tetatoma formation was observed.

Another attractive advantage of direct reprogramming protocols over the use of hPSC-derived transplantations is the ability to generate iNSCs without oncogenes or integrative methods of reprogramming. In fact, two recent studies have successfully reprogrammed mouse embryonic fibroblasts into iNSCs using a chemical cocktail of small molecules without genetic material (Victor et al. 2014; An et al. 2014). These iNSCs differentiated into neurons, astrocytes, and oligodendrocytes when transplanted into the cortices of post-natal and embryonic mouse pups. No tumour formation was detected at 4 weeks post-transplantation.

Until recently, the majority of studies using iNSCs involved transplantation into the cortex, which resulted in the majority of differentiated neurons possessing a glutamatergic identity. For direct reprogramming technologies to be utilised in the treatment of HD, it is essential that the neurons generated in vivo possess the characteristics of functional GABAergic neurons and express markers of MSNs. Furthermore, it is imperative that transplanted cells integrate into the host basal ganglia network. Remarkably, one study has successfully generated such neurons following the transplantation of reprogramming cells derived from adult human fibroblasts into the striatum of immunodeficient mice (Victor et al. 2014). Not only did the transplanted cells differentiate into exceptionally high yields of mature neurons (93% MAP2 positive), the majority of these neurons expressed markers of MSNs (76% GABAergic, 91% DARPP32 positive, 91% FOXP1 positive). Moreover, the transplanted cells demonstrated long-term survival and conversion stability for more than 6 months. Importantly, this study also assessed the functional properties of these neurons using ex vivo electrophysiological recordings. These studies confirmed that the neurons exhibited the majority of the membrane properties characteristic of MSNs. The neurons derived from transplanted cells also exhibited dense dendritic spines and long axon projections from the striatum to the substantia nigra. Together, these results indicated that the transplanted cells and subsequent neurons have functionally integrated into the host neurons network. Ultimately, this may provide evidence for the restoration of striatal function in HD using transplanted iNSCs from patients with HD.

3 Future Challenges of Cell-Based Therapies for Huntington's Disease

While cell replacement therapy trials in animal models have yielded some promising improvement in motor functioning, the potential benefits of these treatments in patients with HD are debatable. A significant and obligatory challenge of transplantation studies is the relevance of the HD model to the human condition. Most studies of HD cell replacement have been conducted using chemical lesion models of the disease (QA and 3-NP). While transgenic HD models may be more representative of the genetic basis of HD, their short lifespans and aggressive phenotypes have seen them selected again in favour of chemical lesion models (Chen et al. 2014; Mattis and Svendsen 2015). This preferential use of chemical lesion models is not without consequence, as these lesions can alter the regional microenvironment of the brain and influence the specific subtype differentiation of transplanted cells (Johann et al. 2007; Gordon et al. 2007, 2009; Chen et al. 2007; Jones and Connor 2011, 2016). As the objective of cell-based therapies for HD is to replenish the depleted striatum, cells would ideally differentiate into functional striatal-located MSNs. However, generating a pure population of MSNs in the host striatum from transplanted cells is unrealistic, prompting the requirement for thorough identity characterisation and migration monitoring of non-target cell types (Chen et al. 2014).

Recent research in the field of somatic cell reprogramming has exposed new avenues for the use of cell-based therapies in the treatment of neurodegenerative disorders. However, unlike largely idiopathic or sporadic neurodegenerative diseases, HD has a definitive genetic basis. Thus, genetic correction of the HD-causative mutation is required before autologous cell transplants can be performed to prevent mHTT production by transplanted cells (Benraiss and Goldman 2011; An et al. 2012, 2014). Although this approach could be successful in the case of regionally exclusive degeneration, the degeneration of MSNs in HD is preferential yet not exclusive, with widespread atrophy of a range of brain regions observable as HD progresses (Ross and Tabrizi 2011). Consequently, the presence of the HD-causative mutation and production of mHTT by all cells in the HD brain may negate any beneficial effects resulting from the production of normal HTT by transplanted cells (Chen et al. 2014). While there may be the opportunity for the autologous transplantation of genetically corrected cells during pre-symptomatic or early disease stages prior to the degeneration of non-striatal brain regions, such interventions would be highly controversial. Ultimately, the progression of cell-based therapies from animal models to human clinical applications is controlled by a wide range of uncertainties surrounding the ability of these therapies to provide a long-term functional benefit which outweigh any adverse effects. As the use of somatic cell reprogramming to generate cells for transplantation is still in its infancy, further research in this area is hoped to generate positive results which can be successfully translated to clinical trials in patients with HD.

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Stem Cell Transplantation for Amyotrophic Lateral Sclerosis

6

Qiang Zhu and Paul Lu

1 Introduction

Amyotrophic lateral sclerosis (ALS), also named as Lou Gehrig's disease, is a motor neuronal degeneration disease, in which the degenerating or death of motor neurons causes loss of control of voluntary muscles (Sreedharan and Brown Jr. 2013). The consequence is weakness of muscles with a wide range of disabilities and eventually death. The cause of motor neuronal degeneration is not known in the majority of ALS patients (sporadic form) except 5–10% patients who have inherited the disease from their parents (familial form).

2 Etiology and Molecular Genetics

In United States alone, approximately 6000 people are diagnosed with ALS each year based on US population studies. This equals to about two people for every 100,000 people across the entire US population. It is estimated that about 20,000 Americans have ALS disease at any given time. According to the ALS CARE Database, 60% of ALS patients are men and 40% women. Most people who developed ALS are middle to elder age (between 40 and 70), with an average age of 55 at the time of diagnosis. However, young adults in their twenties and thirties do get the ALS disease sometimes.

ALS can be divided into two forms: sporadic (sALS) and familial (fALS) (Talbott et al. 2016). Sporadic ALS constitutes the majority of ALS cases in which the disease seems to occur at random with no family history of the disease and even no clearly associated environmental risk factors. The family members of people with sporadic ALS may be at an increased risk for the disease. However, the risk is very low and most of them will not develop ALS.

Familial ALS, on the other hand, constitutes only about 5–10% of all ALS cases. Familial here means that the disease is inherited from an individual's parents, usually from only one parent to carry the gene responsible for the disease. Mutations in Cu/Zn superoxide dismutase 1 (SOD1) was the first gene identified to be associated with ALS (Sangwan and Eisenberg 2016). Up to date more than a dozen genes with mutations have been found to cause familial ALS. Recently, a large hexanucleotide repeat in the noncoding region of an uncharacterized gene named "chromosome 9 open reading frame 72 (*C9ORF72*)" has been linked to both ALS and frontotemporal dementia (FTD). This

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F. Han, P. Lu (eds.), *Stem Cell-based Therapy for Neurodegenerative Diseases*, Advances in Experimental Medicine and Biology 1266, https://doi.org/10.1007/978-981-15-4370-8_6

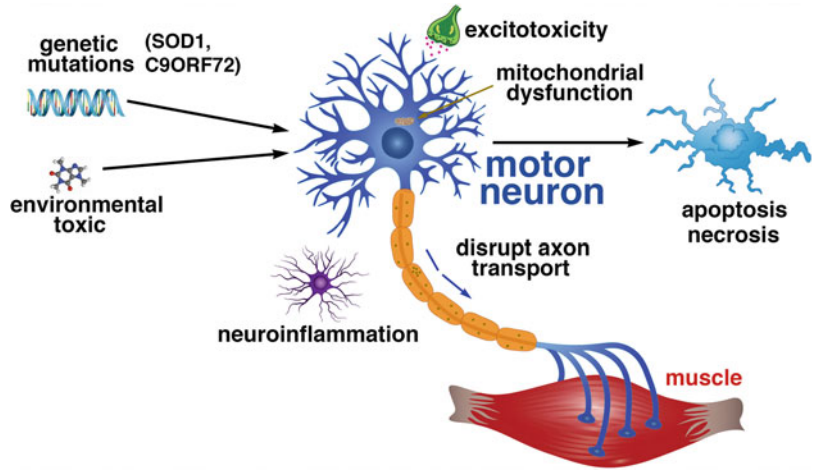

Fig. 6.1 Proposed disease mechanisms of ALS. Both genetic mutations (SOD1 and *C9ORF72*) and environmental toxic play a role in the development of ALS. The cellular character of ALS is motor neuron degeneration by either apoptosis or necrosis. Several mechanisms may contribute to motor neuron degeneration, such as excitotoxicity, neuroinflammation, axon transport disruption, and mitochondrial dysfunction

C9ORF72 mutation affects about 25–40% of all familial ALS cases and is the most common genetic cause for both familiar ALS and FTD (Gitler and Tsuiji 2016). These ALS-associated gene mutations, as well as some unknown environmental factors, relate to various cellular disturbances, such as excitotoxicity, neuroinflammation, axon transport disruption, mitochondrial dysfunction, and synaptic disorders, leading to motor neuron degeneration by apoptosis or necrosis (Fig. 6.1) (Zufiría et al. 2016).

3 Stem Cell Therapy for ALS

Various therapies for ALS have been developed and tested in preclinical animal models and even in clinical trials (Sreedharan and Brown Jr. 2013). One potential therapy, the stem cell transplantation, becomes very attractive since they have potential for neuroprotection and motor neuronal replacement. Stem cells are undifferentiated cells that can self-renew and differentiate into specialized cell lineages. Stem cells can be found and isolated from embryonic and developing tissues or directly isolated from adult tissues. Recent development of induced pluripotent stem cells (iPSCs) are re-programed differentiated adult somatic cells that resemble embryonic stem cells (ESCs) (Takahashi et al. 2007). In developing embryos, stem cells can differentiate into all kinds of the specialized cells—ectoderm, endoderm, and mesoderm. Neural stem cells from neuroectoderm and neural tube can differentiate into all three lineages of neural cells: neurons, astrocytes, and oligodendrocytes. In adult organisms, stem cells and progenitor cells are maintained in certain organs as a repair and replenishing system. The typical example of adult stem cells are bone marrow stem cells consisting of hematopoietic stem cells and some mesenchymal stem cells. Although neural stem cells also exist in adult central nervous system (CNS), they are only in restricted regions of the adult brain, such as subventricular zone and the dentate gyrus of the hippocampus (Paspala et al. 2011).

During stem cell renewal and differentiation, stem cells release many molecules, including many trophic factors and some pro-inflammation cytokines that could protect motor neurons in ALS. This neuroprotective approach could prevent or slow down ALS disease progression.

The key character of ALS disease is motor neuron degeneration. Unfortunately, the CNS neurons cannot regenerate for the replacement of lost neurons. NSCs or neural progenitor cells (NPCs) have potential for neuronal replacement therapy for ALS since they can differentiate into

neurons, especially motor neurons that could integrate into the CNS to re-establish voluntary motor control of muscles.

4 Preclinical Studies of Bone Marrow Stem Cells (BMSCs) or Mesenchymal Stem Cells (MSCs) for ALS

4.1 Sources of MBSCs and MSCs

Bone marrow stem cells refer to the mononuclear fraction of bone marrow cells that comprise most of hematopoietic stem cells and a small amount of mesenchymal stem cells (MSCs) from bone marrow stromal. They may have some hematopoietic progenitors and differentiated bone marrow cells such as lymphocytes and monocytes depending on the isolation methods.

The mesenchymal stem cells not only can be isolated from bone marrow, but also can be derived from adipose tissue, inner organs and blood vessels, and several fetal life support systems, such as amniotic fluid and membrane, umbilical cord, or placenta (Lopez-Verrilli et al. 2016). The increased supply of MSCs definitely supports transplantation studies. However, different sources of MSCs may have different characteristics that influence the repair of neural cells in ALS (Lopez-Verrilli et al. 2016).

4.2 Mechanism of BMSC or MSC Transplant for ALS

One key feature of ALS is the neuroinflammation caused by invasion or reactivation of immune system cells, such as microglia, which can induce A1 astrocytes to promote neuronal death (Liddelow et al. 2017). Studies demonstrate that MSCs have immunomodulation and neuroprotection characteristics and can attenuate activated macrophages/microglia, reduce reactive astrocytes, and increase white matter sparing (Abrams et al. 2009; Osaka et al. 2010; Seo et al. 2011; Ribeiro et al. 2015). This effect of immunomodulation can be achieved not only by local spinal parenchymal transplantation, but also by intravenous (Osaka et al. 2010; Seo et al. 2011) or intrathecal (Cizkova et al. 2011) delivery of MSCs.

4.3 BMSC Transplant in ALS Models

BMSCs are typical adult multipotent hematopoietic stem cells and may contain a small amount of mesenchymal stem cells (MSCs). Some of the early studies, however, reported trans-differentiation of bone marrow–derived cells or MSCs into neuron-like cells, both in vitro (Woodbury et al. 2000) and in vivo (Brazelton et al. 2000; Mezey et al. 2000), even before re-programming adult somatic cells into induced pluripotent stem cells (iPSCs) (Takahashi et al. 2007). These studies lead an early whole BMSC intravenous transplant study to evaluate the potential contribution of BM cells to CNS and mesodermal tissues that are associated with de novo cell generation or as cell fusion (Corti et al. 2004). They transplanted BMSCs derived from green fluorescent protein (GFP) mice, and separately from Thy1-YFP mice in neurons only, into the peritoneal cavity of chimeric Cu/Zn superoxide dismutase (SOD1) mice. Very few recipient neurons become GFP and YFP positive, which were most likely generated by cell fusion (Terada et al. 2002; Ying et al. 2002). In addition, they found participation of BMSCs in striated muscle tissue regeneration since GFP+ myofibrils were present in the heart and skeletal muscles of SOD1 mice. Interestingly, transplanted SOD1 mice showed a significantly delayed disease onset and an increased life span, indicating that transplanted BMSCs provide favorite "non-neuronal" environment (Corti et al. 2004).

The above study demonstrates that mixed chimerism of bone marrow stem cells from health donors can delay the onset and slow the progression of the ALS disease course. In another study, Ohnishi et al. (2009) generated a complete chimerism of the hematopoietic cells by intra–bone marrow to bone marrow transplantation from GFP-transgenic C57BL/6 mice to symptomatic

ALS model (SOD1) mice at an earlier stage of ALS by irradiation of receipt bone marrow. After transplantation, the ALS model mice showed longer survival and slower disease progression than control untreated ones or ALS mice that received BMSCs from the same ALS mouse strain. Interestingly, a significantly high number of GFP(+) cells were found in the ventral horn of the spinal cord where motor neuronal degeneration occurs. Some of these GFP cells become Iba-1-positive microglia. This study suggests that the improvement of the spinal cord environment by the replacement of hematopoietic cells including microglia carrying the mutant gene with normal hematopoietic cells may have neuroprotection effects and thereby slow the progression of the disease.

Besides direct BMSC transplantation in an ALS mouse model, a previous study demonstrated that a hematopoietic growth factor known as granulocyte colony-stimulating factor (GCSF) also has neuroprotective effects in ALS mice model (Pitzer et al. 2008). Therefore, Ohta et al. (2011) examined whether combinatory therapy using BMSC and GCSF have any synergistic therapeutic benefits after disease onset in ALS mice model (SOD1). They transplanted BMSCs by tail vein and injected GCSF every other day for ten times through subcutaneous administration. Interestingly, treatment with BMSCs and GCSF delayed disease progression and prolonged the survival of SOD1 mice, while either treatment alone does not. The mechanism behind this improvement is the reduction of motor neuron loss and the increase of neurotrophin expression, such as glial cell line–derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VGEF), and angiogenin. These studies indicate that combined treatment with BMSCs and GCSF has both neuroprotective and angiogenic effects in ALS mice (Pitzer et al. 2008).

Alternatively, these BMSCs can be activated by stem cell factor (SCF)- or FMS-like tyrosine kinase 3 (flt3) before transplantation (Terashima et al. 2014). In a test, BMSCs were activated with these factors individually for 12 hours in vitro before transplantation. After intravenous transplantation, hSOD1 (G93A) transgenic mice had greater improvement of motor function and survival in the SCF group than in the group receiving naïve BMSCs and the flt3-activated groups. In addition, SCF-activated BMSCs, labeled with GFP, migrated to the spinal cords of recipient mice and expressed the microglia marker Iba1, while the controls received naïve BMSCs or BMSCs treated with flt3 had less amount of Iba1 positive microglia in the spinal cord. These migrating microglia expressed glutamate transporter-1 (GLT-1) that can remove glutamate and potentially reduce glutamate excitotoxicity. Furthermore, mice received SCF-activated BMSCs, reduced the expression of inflammatory cytokines tumor necrosis factor-α and interleukin-1β, and increased the neuroprotective molecule insulin-like growth factor-1 (IGF-1). Therefore, this new strategy of BMSC transplant modulates the character of the migrating donor BMSCs cells and increases their neuroprotective effects (Terashima et al. 2014).

Although the above studies show beneficial effect of BMSC transplantation, most treatments were performed at the presymptomatic stage or onset stage of ALS (Corti et al. 2004; Ohnishi et al. 2009; Ohta et al. 2011; Terashima et al. 2014). To test whether BMSC transplantation can achieve therapeutic effects at symptomatic stages, two groups Gubert et al. (2016) and Venturin et al. (2016) transplanted bone marrow cells in a mouse model of ALS (SOD1 (G93A) at presymptomatic (9–10 weeks old) and symptomatic (14–15 weeks old) stages. Both studies show prolonged survival and delayed disease progression lifespan of the animals when animals received bone marrow transplant at the presymptomatic stage, but not at the symptomatic phases. The lack of beneficial effect of BMSC transplantation at the symptomatic stage should be taken into account in designing clinical trials for ALS.

BMSCs are usually transplanted systemically or intraspinally for the purpose of neuroprotection in brain and spinal cord. However, a recent study showed benefits resulting from the transplantation of BMSCs into hindlimb muscles in a mouse model of motor neuron degeneration (Pastor

et al. 2013). The transplanted mice demonstrated significant improvement in their motor functions, which correlates with an increased number of motor neurons innervating the treated muscle compared with that in the non-treated contralateral symmetric muscle. In addition, transplanted mice express a higher amount of GDNF in the spinal cord compared with control without transplantation. These results suggest that transplantation of BMSCs directly into muscles may have greater potential for motor neuron protection by axonal-guided retrograde neurotrophism. It is interesting to compare muscle transplantation with systemic or intraspinal transplantation for therapeutic effects.

As an alternative to BMSCs, human umbilical cord (hUCB) blood cells contain stem cells similar to hematopoietic stem cells that can be used for ALS. Early studies demonstrates that intravenous administration of a high dose of hUCB cells into SOD1 irradiated mice increased their life span (Chen and Ende 2000; Garbuzova-Davis et al. 2003). The mechanism behind this benefit effect relates to decrease of pro-inflammatory cytokines in the brain and spinal cord and increase of lymphocytes and decrease of neutrophils in the peripheral blood. In addition, microglia density in both cervical and lumbar spinal cords decreases in mice administered with high dose of hUCB cells (Garbuzova-Davis et al. 2003). A late study confirms these beneficial effects by repeat intravenous administration of hUCB in both pre-symptomatical and symptomatical phases of G93A SOD1 mice (Garbuzova-Davis et al. 2012).

Besides systemic administration of human cord blood cells (HuUCB), these cells can be directly administrated into CNS by intracerberoventricular (Bigini et al. 2011) or intrasapinal cord (Knippenberg et al. 2012) injection aiming to improve the potential efficacy of these cells for the treatment of ALS. Both treatments significantly reduced symptom progression in the mouse model of ALS (SOD1G93A mice). The transplanted cells release a series of cytokines and chemokines with anti-inflammatory properties that could be neuroprotective in the mouse models of ALS.

4.4 MSC Transplant in ALS Models

Besides bone marrow derived stem cells that consisted of most hematopoietic stem cells, bone marrow stromal cells or mesenchymal stem cells (MSCs) that are the stem/progenitor cells of skeletal tissues become a very popular stem cell source for ALS due to their immunomodulatory and neuroprotective properties (Hajivalili et al. 2016). MSCs can be easily isolated and cultured from adult bone marrow and are currently in use in clinics for many other diseases.

MSCs have been transplanted systemically into the ALS mouse model (SOD1). Marconi et al. (2013) transplanted adipose-derived MSCs expressing GFP reporter gene isolated from wild-type mice at the clinical onset of ALS, which significantly delayed motor deterioration for 4–6 weeks. There were a higher number of lumbar motor neurons in MSC-treated group compared to phosphate-buffered saline-treated group. This neuroprotective effect correlates with the upregulation of GDNF and bFGF after MSC treatment. When transplanted at the symptomatic stage, MSCs-transplanted mice showed improved survival and motor functions compared with saline-injected controls. However, there are no changes in the number of choline acetyltransferase– and glutamate transporter–type 1-positive cells in MSC transplanted groups compared with controls (Uccelli et al. 2012). In both studies, very few transplanted MSCs home to the central nervous system (CNS), indicating indirect therapeutic effects such as a cross-talk between transplanted MSC with glial cells in the CNS.

MSCs can also be specifically delivered into CNS via intrathecal injection. An early study tested the effects of human MSCs isolated from an ALS patient in SOD1 mice by intrathecal delivery through cisterna magna (Kim et al. 2010). They transplanted three different doses of MSCs: 1×10^4, 2×10^5, and 1×10^6 and found that a high dosage of 1×10^6 cells significantly increases life span and delays motor performance decline. Most transplanted hMSCs spread into the ventricular system and subarachnoid space. Only a small proportion of them migrated into the

spinal cord and brain. The number of motor neurons in this high-dosage-treated group was significantly higher than those of the untreated controls and low-dosage groups. Forostyak et al. (2014) delivered human MSCs intrathecally even at a mid-dosage of 5×10^5 cells at a post-symptom stage of SOD1 rats and transplantation of MSCs reduced disease progression, greatly improved motor activity, and prolonged the survival time of the subjects. In addition, they reported that SOD1 rats have a disorganized abnormal perineuronal net (PNN) structure around the spinal motor neurons, and MSC transplant preserves PNN structure that correlates better survival of motor neurons. The mechanism behind this improvement of survival might be secretion by transplanted MSCs or of modulation of host cells to produce high concentrations of cytokines, such as IL-1α and MCP-1.

4.5 Comparison of Whole BMSC and MSC Transplant for ALS

We reviewed whole BMSC and MSC transplant individually in the preceding sections. Both of these cells have been shown to have therapeutic effects on ALS models. A direct comparison between these two types of cells is necessary to examine which cell type has a better capability for the improvement of motor function in an ALS model. Pastor et al. (2012) analyzed the effect of transplanting whole BMSCs or cultured MSCs isolated from GFP mice into the spinal cord of a motor neuron degenerative mouse model (muscle deficient/osteocondrodystrophy mutation (mdf/ocd). They directly injected one million cells of whole BMSCs and half million cells of MSCs into ventral horn where motor neurons located at L5-S1 region. They then analyzed motor functions using various behavior tests for 7 weeks post-transplantation. They found that whole BMSC-treated mice significantly improved their motor tests, corresponding with a higher GDNF immunoreactivity in the transplanted spinal cord. The transplantation of whole BMSCs from GDNF knockout mice did not elicit motor functional improvement, indicating that donor GDNF expression is necessary for motor neuron survival and functional improvement. MSC-treated mice also have improved motor functions, but to a lesser degree than whole BMSCs. Finally, they demonstrated the stability of BMSC phenotype since isolated engrafted bone BMSCs still can migrate into host spleen, bloodstream, and bone marrow and exhibit bone marrow stem cell morphology after re-transplanted into the spleen of immunodeficient mice. They concluded that transplantation of whole BMSCs is a relatively simple method for the potential treatment of motor neuron degenerative diseases (Pastor et al. 2012).

4.6 Transplantation of Neuronal-Induced MSCs in ALS Models

As a typical adult multipotent stem cells, MSCs have the capacity to differentiate into only mesodermal lineages, such as osteoblasts, chondrocytes, adipocytes, and myocytes (Raff, 2003). However, early studies reported trans-differentiation of bone marrow–derived stem cells or MSCs into neuron-like cells, both in vitro (Woodbury et al. 2000) and in vivo (Brazelton et al. 2000; Mezey et al. 2000). The phenomenon of trans-differentiation of MSCs is explained by the plasticity of adult stem cell (Raff, 2003) or the existence of multipotent adult progenitor cells (MAPCs) (Jiang et al. 2002). Later studies, however, demonstrate that trans-differentiation is an artifact of in vivo cell fusion (Terada et al. 2002; Ying et al. 2002). Similarly, our study, along with others, demonstrate that in vitro differentiation of MSCs into neurons is an artifact of cell stress (Lu et al. 2004; Neuhuber et al. 2004; Bertani et al. 2005).

The introduction of the induced pluripotent stem cell (iPSC) technology really revolutionizes trans-differentiation. Almost any somatic cells, including MSCs, can be re-programmed or converted, into pluripotent stem cells (Takahashi et al. 2007; Barzilay et al. 2009), which then can be induced into neural stem cells (Yuan et al. 2011). In addition, somatic cells, such as bone

marrow cells, can be directly converted to induced neural stem cells (iNSCs) or neurons (iNs) (An et al. 2016). Park et al. (2012) genetically engineered hMSCs to express motor neuron-associated transcription factors, Olig2 and Hb9, and then treated the hMSCs with optimal motor neuron induction medium. More than 30% treated cells expressed motor neuronal markers, ChAT and Islet-1, and manifested the excitable properties of motor neurons. In addition, these induced motor neurons formed functional synaptic connections with muscle fibers in vitro and exhibited characteristics of motor neurons when transplanted into an injured organotypic rat spinal cord slice culture, indicating potential therapy for autologous cell replacement for ALS.

Chan-Il et al. (2013) transduced human MSCs with neurogenin 1 (Ngn1) using retroviral vectors and transplanted one million of MSCs-Ngn1 into the SOD1ALS mouse model by tail vein injection. They found that Ngn1-expressing MSCs exhibited an increase in tropism toward the CNS. When MSCs-Ngn1 were transplanted during pre-symptom stages, they delayed disease onset while the control naive MSCs failed to do so. If MSCs-Ngn1 were transplanted near the onset ages, they elicited motor functions during the symptomatic period even with only a single treatment, while similar motor function improvement requires repeated transplantation of control MSCs. However, there is no data available to verify differentiation of neurons by transplanted MSCs-Ngn1. These results suggest that neural induction or re-programing of transplanted MSCs has a potential benefit for the treatment of ALS.

5 Clinical Trials of Bone Marrow Stem Cells (BMSCs) or Mesenchymal Stem Cells (MSCs) for ALS

5.1 Clinical Trials of Bone Marrow Stem Cells (BMSCs) for ALS

Following observed benefits of whole bone marrow–derived stem cells (BMSCs) in ALS animal models, several clinical studies examined the safety and efficacy of BMSCs transplant in ALS patients. Deda et al. (2009) transplanted mononuclear cells (approximately 300,000 cells in 0.1 mL) derived from bone marrow from health donors into ventral horn of upper cervical (C1–2) spinal cord for 13 sporadic ALS patients that with bulbar involvement and severe loss of movement. In addition, they administrated about ten million cells into gel foam that cover the brain stem and upper cervical spinal cord, five million cells into subarachnoid space, and five million cells intravenously. They reported that nine patients had significant improvement compared with their pre-operative status, confirming by electro-neuro-myography (ENMG) during 1-year follow-up observation. In addition, one patient was stable in his status although three patients died due to lung infection and myocardial infarction after stem cell transplantation (Deda et al. 2009). Notably, the BMSCs were administrated in multiple routes in this trial and the BMSCs stored in gel foam may last long for the release of trophic factors. However, there is no evidence to support this long-lasting effect. In addition, the neurological measurement in this study is questioned by a different research team (Bek et al. 2009).

In another safety study, Blanquer et al. (2012) transplanted autologous bone marrow mononuclear cells (BMNCs) into posterior spinal cord funiculus at T3–4 region in 11 ALS patients. There was not any severe transplant-related adverse event, but 43 non-severe events: 22 events (51%) resolved within 2 weeks, and only four lasted to the end of follow-up. Several ALS functional tests, including forced vital capacity (FVC), ALS-functional rating scale (ALS-FRS), Medical Research Council scale for the assessment of muscle power (MRC), and Norris scales, were not accelerated in the rate of decline post-transplantation. They reported that four patients die post-transplant for reasons unrelated to the transplantation procedure. Pathological analysis demonstrated a greater number of motor neurons survived in the treated segments than the untreated segments. In addition, although motor neurons were surrounded by CD90+ cells, but they did not exhibit deposits of degenerative ubiquitin in the treated segments. This clinical

trial demonstrates not only the safety of intraspinal infusion of autologous BMNC for ALS patients, but also presents evidence of neurotrophic activity of transplanted BMSCs.

In another pilot clinical trial, Prabhakar et al. (2012) delivered autologous bone marrow–derived stem cells through intrathecal infusion in 10 ALS patients. They reported no significant declination in revised ALS Functional Rating Scale (ALSFRS-R) composite score from baseline one-year post-procedure ($P = 0.090$). The median survival time after infusion was 18.0 months and 4-point deterioration median time was 16.7 months. There were no significant adverse events reported. However, there is no control in this pilot study and the follow-up time is relatively short.

Besides these pilot studies, Sharma et al. (2015) transplanted autologous bone marrow mononuclear cell in a retrospective controlled cohort study. They transplanted about 50 million cells intrathecally through lumbar puncture in 37 ALS patients. In addition, they transplanted about 30 million cells intramuscularly at the motor-points of the specific muscles in addition to methylprednisolone, Riluzole, and standard rehabilitation. Twenty control patients received all other treatments except cell transplantation. Statistical analysis demonstrated that the mean survival time of patients in intervention group is 87.76 months, which is higher than control group that survived only for 57.38 months. But it does not reach statistically significant difference ($p = 0.133$). In addition, survival time was significantly higher ($p = 0.039$) in patients with the onset of the disease below 50 years. The intervention group also has higher mean survival duration than the previous epidemiological studies. Besides the standard treatment with Riluzole, combination of BMSCs transplantation and lithium at early intervention may have a positive effect on the survival time for ALS. These findings are promising and need to be verified in a large randomized controlled study using a rigorous methodology.

Since respiratory failure is the main cause of death in ALS patients, Ruiz-López et al. (2016) infused autologous BMSCs intramedullary at thoracic 3–4 level in 11 ALS patients. Although the rapid eye movement (REM) sleep decreased slightly 1 year after the cell transplantation, it does not reach statistically significant comparing to pre-treatment time. In addition, no differences were found in the apnea-hipopnea index, mean oxygen saturation, and nadir desaturation evolution. They concluded that intramedullary injection of MBSCs is safe and does not worsen the cortico-medullar diaphragmatic pathways.

Most above studies transplanted BMSCs targeting on brainstem or spinal cord motor neurons in ALS patients. One study, however, targets on upper motor neurons by transplantation of CD133+ stem cells obtained from peripheral blood into frontal motor cortex (Martinez et al. 2009). The rationale to use CD133+ cells is neuronal differentiation potential. They injected about 2.5–7.5 $\times$ 10^5 CD133+ stem cells into the cortex in 10 ALS patients. The survival of treated patients is statistically higher ($P = 0.01$) than untreated controls. In a follow-up study, the same group performed the same procedure in 67 ALS patients (Martínez et al. 2012) and reported detailed adverse events. The survival is 90% 1 year after transplantation with a mean long-term survival rate of 40.17 months from diagnosis. The occurrence of adverse events is only in a small proportion of patients and only one patient died due to the cell transplantation procedure. They concluded that the autologous transplantation of CD133+ stem cells in the frontal motor cortex is a safe and well-tolerated procedure and may have therapeutic effect for ALS patients.

5.2 Clinical Trials of Bone Marrow Mesenchymal Stem Cells (MSCs) for ALS

Since transplantation of bone marrow–derived mesenchymal stem cells (MSCs) can slow the progression of the disease in ALS animal model, Mazzini et al. (2003) tested the feasibility and safety of intraspinal cord transplantation of autologous MSCs in seven well-monitored patients with ALS. They collected bone marrow from

ALS patients with the standard procedure, and purified and expanded MSCs in vitro. The MSCs were then transplanted into the most central part of spinal cord at T7–9 levels by a micrometric pump injector. No major adverse events were found in transplanted patients, although some minor adverse events occurred, which was reversible after a short period of time. Spinal cord volume appeared normal, and no signs of abnormal cell proliferation were observed. In two follow-up studies, they additionally observed that the linear decline of the forced vital capacity is significantly slowing down in half patients (8 out of 16) 3–4 years post-MSCs transplantation (Mazzini et al. 2006, 2008). They concluded that the procedure of autologous MSC transplantation is safe and well tolerated by ALS patients and the clinical results seem encouraging (Mazzini et al. 2003, 2006). In a late long-term study up to 9 years post-transplantation, however, they observed no clear clinical benefits (Mazzini et al. 2012, 2015). The effect of early slow progression of disease observed may be transient and does not last.

MSCs can also be transplanted intrathecally for the spread of cells along cerebrospinal fluid. Syková et al. (2017) transplanted $15 \pm 4.5 \times 10^6$ of autologous MSCs via lumbar puncture into the cerebrospinal fluid in 26 ALS Patients. They observed no suspected serious adverse reactions after cell transplantation. They found a reduction in the ALS functional rating scale (ALSFRS) decline at 3 months post-transplantation, but it did not last in all patients. In addition, they found that forced vital capacity (FVC) values remained stable or above 70% for a time period of 9 months; FVC and values of weakness scales (WS) were stable in 75% of patients at 3 months post-transplantation. They concluded that the intrathecal transplantation of MSCs is a safe procedure, which can slow down the ALS disease progression.

Since the effect of single intrathecal transplantation of autologous MSCs is limited, Oh et al. (2015) evaluated the safety of two repeated intrathecal injections of autologous MSCs in 8 ALS patients. Autologous MSCs were isolated two times from ALS patients' bone marrow at an interval of 26 days and were then expanded in vitro for 28 days. Seven patients received 2 intrathecal injections of autologous MSCs (1×10^6 cells per kg) 26 days apart through standard lumbar puncture. They found no serious adverse events, neither therapeutic effect, during the 12-month follow-up period, indicating intrathecal injections of autologous MSCs were safe and may be feasible for the treatment of ALS patients (Oh et al. 2015).

Since lumbar injection of MSCs could tend to sink downward rather than ascending to thoracic and cervical spinal cord and brain, Baek et al. (2012) tested whether intraventricular injection of MSCs could be feasible. MSCs were isolated from the bone marrow of a male ALS patient as autologous MSCs and were expanded in vitro. They were directly injected into the ALS patient's lateral ventricle via the Ommaya reservoir at a dose of 1×10^6 cells/kg. They observed no serious adverse events associated with the stem cell therapy. However, no result was reported about the distribution of transplanted MSCs in CNS (Baek et al. 2012).

The MSCs were transplanted autologously in the above studies, which is the ideal transplantation for ALS as it avoids immunosuppression treatments that may further complicate the general health of ALS patients. But a key question is whether MSCs isolated from ALS patients have the same immunomodulatory and neuroprotective properties as those from health donors. Ferrero et al. (2008) compared MSCs isolated from sporadic ALS patients with MSCs from healthy donors. Growth kinetics, immunophenotype, telomere length, and karyotype were evaluated at the third passages of culture. MSCs isolated from health donors have a slightly faster population doubling time than those from ALS patients. But there was no difference between donors and patients in the immunophenotype analysis, neither chromosomal alteration nor evidence of cellular senescence. In addition, both donor and patient MSCs have similar differentiation potential into adipocytes, osteoblasts, chondrocytes, and neuron-like cells after exposure to specific conditioning media. These results suggest that MSCs isolated from ALS patients can be

extensively expanded without any functional modification of the cells and could be used for autologous transplantation.

However, in a following-up study, Tichon et al. (2009) detected telomerase activity, telomerase enzyme protein, and telomerase RNA transcripts from hMSC-derived from ALS but were not in hMSC from the healthy donors. Since high telomerase activity is detected in nearly all human cancers, the expression of telomerase in hMSC derived from ALS patients may impose risk when transplanted autologous into ALS patients themselves (Kumar et al. 2016). Besides telomerase activity, Cho et al. (2010) demonstrated significant reductions of the expression of Oct-4 and Nanog (two pluripotent stem cell markers), and of the trophic factors, such as ANG, FGF -2, HGF, IGF-1, PIGF, SDF-1alpha, TGF-beta, and VEGF, in MSCs isolated from ALS patients comparing to MSCs from health donors, indicating diminished stem cell capacity from ALS patients. Furthermore, the same group showed that the functional deficiency of BMSCs in ALS patients is proportional to their disease progression rate and suggests healthy allogeneic MSC transplantation for ALS patients (Koh et al. 2012). The same group also tests whether the functions of MSCs from ALS patients can be restored through the inhibition of DNA methyltransferase in recognition that there is a high association of alteration of DNA methylation with aging and neurodegenerative disorders (Oh et al. 2016). The treatment of MSCs from ALS patients with the DNA methyltransferase inhibitor, RG108, increased the expression of the anti-senescence genes TERT, VEGF, and ANG, and decreased the expression of the senescence-related genes ATM and p21. In addition, RG108-treated MSCs derived from ALS patients reduced the activity of SA-β-galactosidase and the expression of senescence proteins p53 and p16. Furthermore, RG108 treatment improved the cell migration ability and protected against oxidative damage in ALS-MSCs. These results suggest that the functions of MSCs derived from ALS patients can be restored by inhibiting excessively expressed DNA methyltransferase.

MSCs obtained from bone marrow need to be expanded to sufficient number in vitro in order for transplant. This is especially true for systemic transplantation where a large number of MSCs are needed. The same Korea group isolated and expanded MSCs from ALS patients for analyzing the growth kinetics, differentiation potential, cellular surface antigen expression, karyotype modifications, and cytokine secretion during long-term culture (Choi et al. 2010). MSCs at early passages have higher growth rate than at late passages. The highest growth rate is in the third passage. The cell surface antigens and the karyotype of the MSCs are stable from the first to the tenth passage. In addition, secretion of IL-6, VEGF and IL-8, IL-15, GM-CSF, IL-10, PDGF-bb, G-CSF, IL-1beta, basic FGF and IFN-gamma in culture medium gradually decreases over prolonged culture. Therefore, the authors suggest that MSCs at earlier passages might be an optimal stage for stem cell therapy for ALS patients (Choi et al. 2010).

Although the above studies indicate ALS patients' own MSCs may have telomerase activity (Tichon et al. 2009), diminished stem cell capacity that is proportional to disease progression rate (Cho et al. 2010; Koh et al. 2012), few clinical studies test the allogenic transplantation of MSCs from health patients to ALS patients. This is probably due to the potential of complication from immunosuppression if transplanted allogenically. It is also possible to transplant human leukocyte antigen (HLA)–matched MSCs from a health donor to an ALS patient.

Besides naïve MSCs, one study attempted to induce human MSCs into neural stem cells and then transplanted them intraspinally into ALS patient (Nafissi et al. 2016). They induced human MSCs with bFGF and EGF through neurosphere stage, but not by re-programming with transcription factors. The induced neural stem cells were verified only by simple immunocytochemistry, but not by gene profile analysis, in vitro differentiation and electrophysiology. After transplantation in 8 ALS patients, none of the patients had perioperative mortality or major morbidity. One patient died due to pulmonary embolism 12 months post-transplantation.

Although patients were stable in the first several months, their conditions deteriorated afterward, indicating no lasting effect of transplanted so-called neural stem cells. Whether the transplanted so-called "neural stem cells" are real neural stem cells that can differentiate into both neurons and glia in vivo is unknown.

6 Preclinical Studies of Neural Stem Cells (NSCs) for ALS

6.1 Sources of NSCs

Neural stem cells (NSCs) are multipotent stem cells in the nervous system that can self-renew and generate the neurons and glia during development and in certain neurogenic regions in adult central nervous system (CNS). NSCs enter an intermediate stage called neural progenitor cells (NPCs) that gradually specify as neuronal or glial linage restricted precursor cells, such as neuronal-restricted precursors or glial-restricted precursors that differentiate into neurons and glia, respectively (Mayer-Proschel et al. 1997; Rao and Mayer-Proschel 1997). Traditionally, NSCs or NPCs can be obtained from developing (fetal) CNS tissue. These cells can also be isolated from adult CNS tissues that are neurogenic, including the subventricular and subgranular zone (Mathieu et al. 2010).

Besides from developing or adult CNS tissues, NSCs/NPCs can be generated from pluripotent embryonic stem cells (ESCs) derived from the inner cell mass of mammalian embryos at the blastocyst stage. ESCs can be induced into NSCs/NPCs that can be further specified into different phenotypes of neurons or glia using various cell-signaling molecules (Li et al. 2011). Similarly, NSCs/NPCs can be generated from induced pluripotent stem cells (iPSCs), which are adult pluripotent stem cells generated from somatic cells by reprogramming factors (Takahashi et al. 2007).

By using distinct sets of transcription factors, or downregulating polypyrimidine-tract-binding (PTB) protein, it is possible to directly reprogram somatic cells, such as fibroblasts, toward a generic neuronal phenotype or NSCs (Mertens et al. 2016; Xue et al. 2016). Furthermore, this direct conversion approach can generate induced motor neurons (iMNs), which acquired the motor neuron identity evidenced by the program of gene expression, electrophysiological activity, synaptic functionality, in vivo engraftment capacity, and sensitivity to ALS disease stimuli, from human fibroblasts, suggesting potential clinic applications in motor neurodegenerative diseases (Son et al. 2011).

6.2 Mechanism of Neural Stem Cell Transplant for ALS

6.2.1 Cell Replacement Strategies

Since motor neurons are selectively degenerated and vulnerable to cell death in ALS, replacement of motor neurons by the transplantation of neural stem cells or motor neuronal progenitor cells appears to be direct and could be efficient. Previously, the isolation of enough motor neurons or their progenitors from fetal spinal cord for transplantation is a real challenge since motor neurons do not divide and there is no surface antigen for the isolation of motor neurons or their progenitors from whole spinal cord-derived neural cells. Recently, motor neurons or their progenitors can be generated in vitro from pluripotent stem cells, including embryonic stem cells (ESCs) (Wichterle et al. 2002; Li et al. 2008; Adams et al. 2015; Cortés et al. 2016), induced pluripotent stem cells (iPSCs) (Dimos et al. 2008; Karumbayaram et al. 2009; Sances et al. 2016), or directly from somatic cells as induced motor neurons (Son et al. 2011; Liu et al. 2016; Zhang et al. 2017). These motor neurons or their progenitors, however, are generated mostly for modeling of ALS disease, especially when they are derived from iPSCs or induced motor neurons that can be reprogrammed from ALS patients (Son et al. 2011; Liu et al. 2016; Sances et al. 2016; Zhang et al. 2017). Very few studies attempt to transplant these motor neurons or their progenitors for motor neuronal replacement therapy.

Besides neuronal replacement therapy, recent studies demonstrate that astrocytes from both familial and sporadic ALS patients or transgenic mice are toxic specifically to motor neurons (Chen et al. 2015; Qian et al. 2017). Therefore, glial replacement could potentially reduce motor neuron degeneration for ALS. Several studies, including us, show that transplanted human NSCs or glial progenitors can re-populate into large numbers of astroglia and migrate long distance in rodent CNS (Han et al. 2013; Mormone et al. 2014; Chen et al. 2015; Lu et al. 2017). More importantly, human graft–derived astrocytes can replace host astrocytes and integrate into host CNS, enhancing both activity-dependent plasticity and learning in mice. Previous studies demonstrate that transplantation of either rodent or human glial-restricted precursors (GRPs) survive, integrate, and differentiate into both astrocytes and oligodendrocytes in the spinal cord of rodent models of ALS (Lepore et al. 2008, 2011). Transplanted rodent GRPs restore certain diaphragmatic function (Lepore et al. 2008). However, in a follow-up study, transplantation of human GRPs failed to provide motor neuron protection or any therapeutic benefits on functional outcome measures (Lepore et al. 2011).

6.2.2 Neuroprotection Mechanism

Besides the functional properties of NSCs that differentiate into neurons and glia, the grafted NSCs and their derived neural cells support the survival of damaged MNs through secretion of neurotrophic factors and anti-inflammatory activities. NSCs and their derived neural cells produce and excrete different kinds of immunomodulatory molecules that regulate cell growth, migration, and differentiation, including neurogenesis and angiogenesis (Czarzasta et al. 2017). One study demonstrates that vascular endothelial growth factor (VEGF) released from NSC-treated transgenic animals promoted a neuroprotective effect by the expression of anti-apoptotic and cell lifespan-mediating molecules, and downregulation of pro-apoptotic proteins. In addition, transplanted cells support the survival of motor neuron and improve motor function in ALS models (Hwang et al. 2009).

In addition, NSCs express and release neurotrophic factors from their original transplanted stem cells and their neuronal progeny (Haidet-Phillips and Maragakis 2015). Our study shows that NSCs naturally and constitutively secrete significant quantities of several neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line–derived neurotrophic factor (GDNF) both in vitro and in vivo (Lu et al. 2003). In addition, grafted NSCs support extensive growth of host axons including axons from ChAT positive motor neurons after spinal cord injury. Those growing host axons are known to be sensitive to growth factors; and among these growth factors, BDNF, CNTF, IGF-1 and GDNF play critical roles in motor neuronal survival (Henriques et al. 2010).

NSCs can be engineered to over-express neurotrophic factors to support motor neuronal survival. Some pioneering research showed that human neural progenitor cells transduced by lentiviral vectors to express and secrete GDNF were able to integrate into the spinal cords of SOD1G93A rats after intraspinal transplantation (Klein et al. 2005). Further studies demonstrated that this approach leads to the protection of motor neurons (Suzuki et al. 2007) and maintain respiratory function in ALS model (Nichols et al. 2013).

Furthermore, like other stem cells, both NSCs and neural precursor cells can release soluble molecules and express immunorelevant receptors that modulate the environment of inflammation. This "bystander" immunomodulation has therapeutic potentials to regulate inflammation and to facilitate resident cells for tissue repair (Teng et al. 2012).

6.3 Neural Stem or Precursor Cell Transplant for ALS in Animal Models

6.3.1 Neural Stem Cells or Precursor Cells from Fetal CNS Tissue

Before ESCs and iPSCs become popular, the main source of NSCs is from fetal CNS tissue.

NSCs derived from fetal CNS tissue can be cultured and maintained at stem cell stage using growth factors such as bFGF and EGF (Shihabuddin et al. 1999). For motor neuron replacement therapy in ALS models, the generation of cholinergic motor neurons is important. In an early study, Wu et al. (2002) treated human fetal brain–derived NSCs with those important growth factors or chemicals for the induction of cholinergic neurons, including bFGF, EGF, LIF, sonic hedgehog amino-terminal peptide (Shh-N), all-trans retinoic acid (RA), NGF, BDNF, NT-3, and natural mouse laminin and heparin. With this in vitro priming procedure, almost all fetal brain–derived NSCs can differentiate into pure population of neurons after being transplanted within adult rat CNS. Furthermore, the grafted cells differentiated into cholinergic positive motor neurons in a region-specific manner, especially in the spinal cord ventral horn where motor neurons lost in ALS patients. The generation of cholinergic positive motor neurons brings neuronal replacement therapy closer for the ALS treatment.

In a follow-up study, the same group demonstrated that grafted human NSCs not only differentiate into motor neurons in rats with chronic motoneuron deficiency, but more importantly, human NSCs–derived motoneurons extend their axons passing through ventral roots and sciatic nerve for neuromuscular junction formation (Gao et al. 2005). Notably, 51% grafted human cells are cholinergic and Hb9 positive, and 19% of them can be retrogradely labeled from muscle. Furthermore, they showed a partial improvement of motor function that correlates with new cholinergic innervations.

Although the above studies suggest potential motor neuronal replacement, the host animals used are not ALS models. Corti et al. (2007) used a sub-population of NSCs double positive for Lewis X and the chemokine receptor CXCR4 (LeX1 CXCR4) from mice expressing GFP in all tissues or only in motor neurons (HB9-GFP mice) and primed them with morphogenetic stimuli. They transplanted these primed NSCs into a commonly used ALS model of SOD1-G93A transgenic mouse. Histology analysis showed that great integration of grafted NSCs and most of them differentiate into neurons. More importantly, as high as 76% grafted cells from HB9-GFP mice differentiated into ChAT-positive motor neurons–like cells and could constitute about 20% of total MNs in transplant region of lumbar spinal cord. In addition, they demonstrated that both VEGF- and IGF1-dependent pathways are significantly modulated in transplanted animals when compared to controls, indicating some neuroprotection. Furthermore, mice that received NSC transplant exhibited a delayed onset and progression of disease and survived significantly longer than control animals by 23 days (Corti et al. 2007).

In a pre-clinical study carried out by Neuralstem Inc. and its collaborators, human fetal spinal cord–derived NSCs were transplanted into the ventral horn in both the lumbar (L4–L5) and cervical (C4–C5) spinal cord of SOD1 G93A rats to test whether it is feasible for this dual grafting paradigm to target muscle groups in both forelimb and hindlimb (Xu et al. 2011). They demonstrate that rats that received NSC graft at the two spinal regions lived 17 days longer compared to controls that received dead NSCs in the similar positions. In addition, the onset of the disease was delayed by 10 days compared to control animals. Histology analysis showed survival and differentiation of neurons from transplanted human NSCs. Although this study shows positive potential of NSC transplant, the transplantation is done prior to the onset of motor neuron disease, which may not apply to clinical treatment of ALS patients.

To further analyze the degree of therapeutic effects of hNSC graft in symptomatic SOD1G93A rats, human NSCs were transplanted into the lumbar spinal ventral horn again to assess whether functional integrity of the descending motor system was present in symptomatic ALS rat models (Hefferan et al. 2012). Histological study showed that grafted hNSCs into the lumbar spinal cord of SOD1G93A rats protect α motoneurons that are surrounding the grafted cells, but not protect α motoneuron pools away from lumbar segments that received grafts. Indeed, there is a near-complete loss of

descending motor tract conduction by motor-evoked potentials recorded from the thoracic spinal cord, indicating that local transplantation of NSCs alone may not be enough to restore functional integration and connectivity of motor neurons with their supraspinal partners.

In addition to the intraspinal transplantation, these fetal CNS–derived NSCs can be transplanted intrathecally into the CNS (Lee et al. 2014) or even intravenously (Mitrecić et al. 2010). The intrathecal or intravenous transplantation has advantages such as minimal invasion and potential widespread of transplanted cells in ALS models. However, the survival and integration into CNS, especially into motor neuron pool regions, are questionable. After intrathecal transplantation of immortalized human NSCs in the lumbar region of the SOD1G93A mice, some human cells, including the ones expressing motor neuron–specific markers ChAT and HB9, did integrate into the host spinal cord. However, the number of differentiated motor neurons is moderate. Even so, the onset of clinical signs in ALS mice was delayed for 7 days and the animal life span was extended significantly for 20 days after transplantation. These therapeutic benefits may not only come from some neuronal replacement, but also from neuroprotection provided by transplanted cells that circulate in host CNS.

6.3.2 Neural Stem Cells or Precursor Cells from ESCs

Since ESCs are pluripotent stem cells that can differentiate into almost any cell types, including NSCs, and have unlimited capacity for self-renewal. Therefore, they have great therapeutic potential for regenerative medicine and tissue replacement after injury or disease, including motor neuronal replacement for ALS.

Harper et al. (2004) generated spinal cord motor neurons from mouse ESCs to determine if they can replace motor neurons in the adult mammalian spinal cord. They transplanted motor neuron–committed ESCs into the adult rat spinal cord with motor neuron injury by intracranial inoculation of rat-adapted neuroadapted Sindbis virus and find that about 3000 ESCs-derived motor neurons survived in the spinal cord of each animal 1 month post-transplantation. However, they found that ESC-derived axonal growth was inhibited by white matter myelin, which is in contrast to our recent studies showing the enhancement of axonal growth in adult host white matter (Lu et al. 2012, 2014a, 2017; Poplawski et al. (2018). Nevertheless, they found that treatments with dibutyryl cAMP (dbcAMP) or a Rho kinase inhibitor can overcome this inhibition. Importantly, they found that 80 ESCs-derived motor axons extending into the ventral roots of each animal after the infusion of dbcAMP. These results demonstrate that ESC-derived NSCs can replace motor neurons in motor neuron–degenerated diseases, including ALS.

To test whether transplanted motor neurons can replace or prevent motor deterioration in transgenic SOD1G93A rats, mouse ESCs were differentiated to motor neurons expressing GFP under the motor neuron–specific gene HB9 promoter and were grafted into the lumbar spinal cord of adult wild-type (WT) or SOD1G93A rats at 10 weeks of pre-symptomatic stage (López-González et al. 2009). Grafted cells with motor neuronal phenotype can survive for at least 1 week in hSOD1G93A animals. However, no grafted GFP+ neurons, neither endogenous ChAT+ motor neurons, survived in sham and grafted SOD1G93A rats, which was in contrast to that in wild-type rats that received grafted GFP-positive motor neurons in their spinal cords at the same age. The loss of both endogenous and grafted motor neurons correlates sudden decrease of motor performance from week 16 onward. These results indicate that the environment of transgenic hSOD1G93A is detrimental to both endogenous and grafted motor neurons in the long term, raising the concern that the direct replacement of lost motor neurons can reverse the course of ALS.

6.3.3 Neural Stem Cells or Precursor Cells from iPSCs

Induced pluripotent stem cells (iPSCs) have great potential as a resource for the generation of NSCs/NPCs, including motor neuronal progenitors, since they are similar to ESCs but

can be generated from adult somatic cells. In addition, iPSCs can be potentially transplanted autologous to avoid the complication of immunosuppression. Popescu et al. (2013) transplanted human iPSC-derived NPCs into the spinal cord of wild-type and SOD1G93A transgenic rats and studied the survival and differentiation of human NPCs until 60 days post-transplantation. They showed that human NPCs survived and engrafted in the adult spinal cord. Human NPCs differentiated into neurons started at day 30 and gradually mature as MAP 2+ neurons. However, the number of grafted human cells reduced over time, especially in SOD1(G93A) rats. There was no astroglial differentiation at this short time period. There is no clear evidence to show maturation of ChAT-positive motor neurons from these transplanted human NPCs. In addition, the axonal growth or synaptic formation from grafted neurons was not reported, and neither were functional outcomes after engraftment, such as deficits of motor function or progression of the disease (Popescu et al. 2013).

Nizzardo et al. (2014) tested a minimal invasive approach by intrathecal or intravenous injection of human iPSC-derived motor NPCs into ALS mice model. The NPC population isolated has high aldehyde dehydrogenase activity, low side scatter, and integrin VLA4 positivity that allows them to cross the blood-brain barrier (BBB). To compensate the loss of transplanted cells, they repeatedly injected human motor precursor cells into the host mice (3x intrathecal and weekly (7x) for intravenous). Interestingly, transplanted NPCs migrated and engrafted into the host CNS through both routes of injection. ALS mice treated with motor precursor cells had better neuromuscular function, motor unit pathology, and extended life span significantly than controls. The mechanisms behind these improvements are related to neurotrophic factor production and microgliosis and macrogliosis reduction. These results suggest that systemic or intrathecal administration of iPSC-derived NSCs, or NPCs can be an effective approach to treat ALS (Nizzardo et al. 2014).

In a follow-up study, the same group tests a subpopulation of iPSC-NSCs positive for LewisX-CXCR4-*b*1-integrin for their therapeutic effect on the ALS mice model (Nizzardo et al. 2016). They again injected NSCs intrathecally into transgenic SOD1G93A and found survival of grafted and GFP+ cells in host spinal cord parenchyma. In addition, ALS mice that received NSC transplant have better neuromuscular junction integrity compared to control. Furthermore, the authors claimed that grafted NSCs induce novel axonal sprouting and reduce macro- and microgliosis. The grafted mice survive significant longer (23 days) than controls. These results indicate that grafted NSCs have neuroprotection effect on ALS mice (Nizzardo et al. 2016). These studies, however, do not address whether grafted NSCs can replace lost motor neurons in the host spinal cord.

Since astrocyte replacement could be a feasible therapy for ALS (Lepore et al. 2008, 2011), Kondo et al. (2014) generated glial-rich neural progenitors (GRNPs) from human iPSCs by the stimulation of the LIF/BMP signaling. They transplanted GRNPs into the lumbar spinal cord after the onset of ALS phenotype in the mouse model. The majority (about 70–80%) of transplanted cells differentiated into astrocytes. In addition, the transplantation of GRNPs increases neurotrophic factor production from host and activates AKT signal that is downstream from the VEGF signal and promotes cell growth and survival in ALS. Furthermore, ALS mice that received GRNP transplant showed prolonged lifespan (7.8% and about 12 days) compared to control. This prolonged lifespan is modest compared to previous studies since most of them did cell transplant before disease onset in ALS models.

7 Clinical Trials of Neural Stem Cells for ALS

The rapid proliferation of ESCs and iPSCs and their pluripotency could lead to teratoma or tumor formation even after their differentiation into NSCs or NPCs, since there could be some contaminated original pluripotent stem cells. This is especially true for iPSCs since they are

generated by reprogramming of adult somatic cells into pluripotent stem cells with four transcription factors, one of which, the Myc, is a proto-oncogene (Takahashi et al. 2007). Several recent studies report the over-growth or tumor formation following the transplantation of human iPSC-derived NSCs in spinal cord injury models and other neurological disease models (Miura et al. 2009; Koyanagi-Aoi et al. 2013; Nori et al. 2015; Katsukawa et al. 2016; Okubo et al. 2016). The mechanisms behind this overgrowth are probably reactivation of the initial silenced transgenes (Choi et al. 2014), instability of adult genome (Ruiz et al. 2015), or generation of primitive NSCs that divide aggressively for a long term (Nori et al. 2015; Katsukawa et al. 2016; Okubo et al. 2016). The risk of graft expansion and tumor formation may limit or prevent clinical translation of ESC- or iPSC-derived NSCs for the treatment of ALS and other neurological diseases. Therefore, most clinical trials test the primary NSCs derived from developing (fetal) CNS.

Since there is a good progress in preclinical studies for NSC-based therapies, the US Food and Drug Administration approved a clinical trial for the transplantation of human fetal spinal cord–derived NSCs generated by Neuralstem Inc. using the intra-spinal injection method. In this phase I clinical trial, 18 patients with ALS received fetal NSC transplantation and were monitored for up to 2.5 years. Among these 18 patients, 12 patients received unilateral or bilateral microinjections into the lumbar spinal cord (Riley et al. 2012), and six received unilateral microinjections into the cervical spinal cord (Feldman et al. 2014). Three of the second cohort of six subjects also received bilateral lumbar injections as part of the earlier trial cohort. Therefore, the second cohort of subjects consists of both single target of cervical cord and dual target of both lumbar and cervical cord. There were no observed major adverse events attributable to the surgery or cells (Riley et al. 2012; Feldman et al. 2014). The authors compared postsurgical outcome data to predicted outcome points extrapolated from pre-surgical disease progression slopes and found some improvements in several measures at 6, 9, 12, and 15 months after surgery. Notably, there are two separate positive slopes observed in those 3 subjects that received dual targets of cell graft, which correlates to the times after each cell grafting. This phase I trial indicates that intra-spinal transplantation of spinal cord–derived NSCs in ALS patients is feasible and well tolerated and provides base for future trials.

The same group conducted a phase II trial following the above phase I trial (Glass et al. 2016). Fifteen participants received increased dosage of cells or increased injection sites. They all received bilateral injections into the cervical spinal cord, and one group received dual injections into both cervical and lumbar cord. They assessed adverse events and progression of disease, forced vital capacity, and quantitative measures of strength compared to three separate historical control groups. There are no differences in mean rates of progression between cell transplant groups and the historical controls. The only conclusion is that the high dosage of neural stem cells can be transplanted in ALS patients, including dual targets of both cervical and lumbar cord (Glass et al. 2016).

In addition, the same group attempted to identify whether transplanted human NSCs survived in the spinal cord in patients with ALS (Tadesse et al. 2014). Six participants survived from 196–921 days post-transplantation. The analysis of genomic DNA from donor cells reveals that maximum percentage of donor DNA in each case ranges from 0.67% to 5.4% of total DNA. Fluorescence in situ hybridization identifies XY chromosomes–positive donor cells in a female recipient, confirming the survival of donor cells for 196 days after transplantation. In addition, some XY-positive donor cells are labeled for the neuronal marker NeuN and stem cell marker SOX2. The existence of SOX2-positive cells suggests that some grafted cells still maintain at their stem cell stage 6 months after transplantation.

Besides the US trials, an Italy group reports initial results from a recent phase I clinical trial for ALS (Mazzini et al. 2015). They generated human NSCs from natural death of fetus brains but offer no rationale why to choose brain-

derived NSCs instead of spinal cord–derived NSCs.

The brain-derived NSCs were transplanted into lumbar spinal cord in six patients. There is no disease progression increase related to cell transplantation, and three patients show some transient improvement, including the sub-score ambulation on the ALS-FRS-R scale and the MRC score for tibialis anterior. They conclude that transplantation of fetal brain–derived NSCs in ALS patients is a safe cell therapy approach.

8 Enhancement of Neural Stem Cell Survival in Spinal Cord (Our Own Works)

One of the critical problems with stem cell transplantation, especially NSCs or NPCs, for ALS and other neurological conditions is the survival of transplanted cells. This is especially true when NSCs and NPCs are transplanted into lesioned or degenerated CNS where the micro-environment is unfavorable to survival and differentiation of transplanted cells (Medalha et al. 2014).

Our group recently developed a methodology to enhance survival and differentiation of grafted NSCs or NPCs in spinal cord injury models (Lu et al. 2012, 2014b; Hou et al. 2013). We used fibrin matrixes to retain grafted cells in the lesioned spinal cord and a growth factor cocktail that consists of nine growth factors and a cell death inhibitor to support their survival and differentiation (Lu et al. 2012, 2014b). These growth factors and a cell death inhibitor can be classified into four categories: (1) Both bFGF and EGF promote proliferation of neural stem cells or their progenitor cells (Tarasenko et al. 2004); (2) BDNF, NT-3, GDNF, IGF-1, aFGF, and bFGF all have neuroprotective properties on neuronal progenitors and neurons; (3) VEGF, aFGF, PDGF, and HGF promote angiogenesis (Kuwano et al. 2001), which is important for graft vascularization that supports their survival; (4) MDL28170 is a calpain inhibitor that reduces apoptosis and cell death and promotes grafted Schwann cell survival in a spinal cord injury model (Hill et al. 2010). Our protocol results in the retention of grafted neural stem cells or progenitor cells in the fibrin matrixes and significantly supports their survival within the lesion site. The grafted cells completely filled the large lesion cavity, integrated well with the host, and differentiated into a large number of neurons and glia, including ChAT-positive motor neurons (Fig. 6.2a, b). Interestingly, motor neurons formed cluster in the graft, partially resembling their naïve distribution (Fig. 6.2b). Most remarkably, great numbers of axons were derived from the grafted neurons and extended into host spinal cord and brain both rostrally and caudally for long distance (Fig. 6.2c). Graft-derived motor neurons also extended their motor axons into ventral roots that were close to the graft (Fig. 6.2d), indicating proper targeting potential. These results indicate that our protocol transforms the initial inhospitable adult lesion environment into one that is highly permissive for supporting survival, growth, and differentiation of NSCs. Furthermore, some of these graft-derived axons are remyelinated and exhibit extensive synaptic connectivity with host neurons (Lu et al. 2012; Hunt et al. 2017). On the other hand, host supraspinal axons, including corticospinal tract axons, regenerated into graft for connectivity (Lu et al. 2012, 2014a, 2017; Kadoya et al. 2016). Novel functional and electrophysiological relays were formed from grafted neurons across complete spinal transection sites, resulting in the improvement of behavioral tests (Lu et al. 2012; Kadoya et al. 2016). These results suggest that neuronal replacement can be achievable and could be used to replace degenerated motor neurons for ALS patients.

Besides neuronal replacement, grafted *human* NSCs in rodent spinal cord injury models differentiate into glia, especially astrocytes, that can migrate into host spinal cord for long distance (Chen et al. 2015). Interestingly, graft-derived astrocytes can replace host astrocytes over a long period of 9 months. The human graft–derived astrocytes form networks through their processes, encircle endogenous neurons, and extend end feet that wrapped around blood vessels without altering locomotion (Chen et al. 2015). We observed similar astrocyte migration

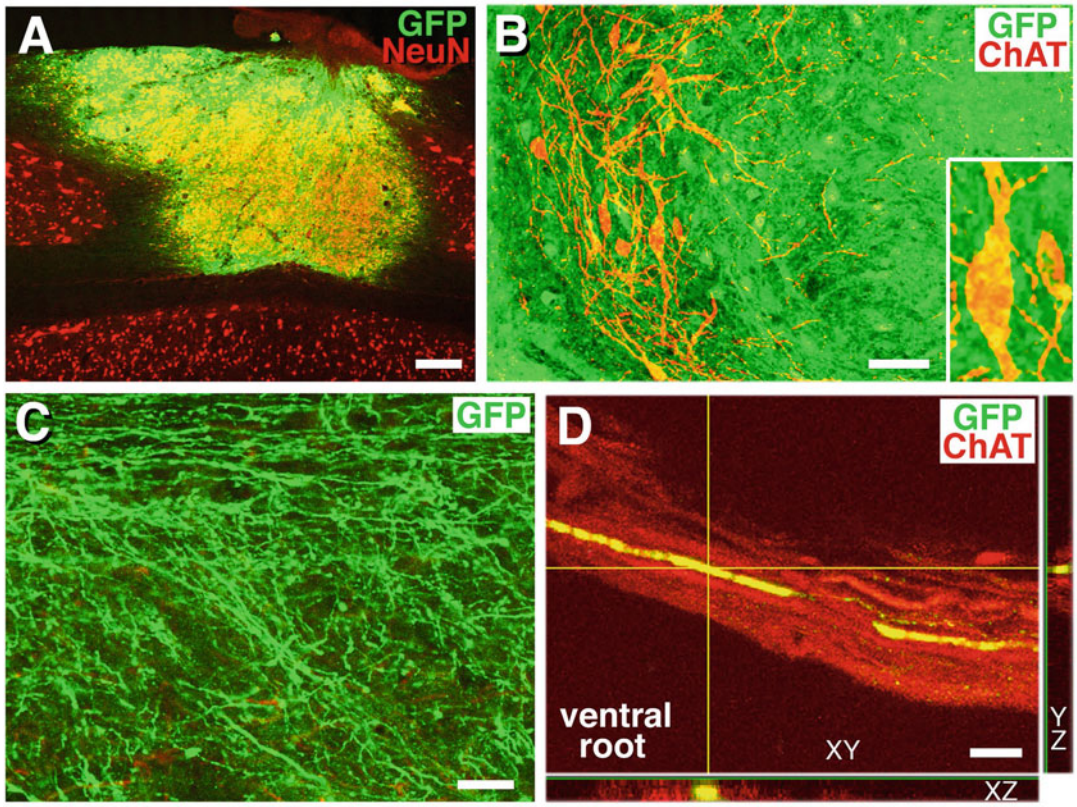

Fig. 6.2 Survival, Neuronal Differentiation, and Axonal Outgrowth of Grafted NPCs in Spinal Cord Injury Site. (**a**) Double labeling of *GFP* and *NeuN* reveals survival, filling of C5 hemisection injury site, and differentiation of *NeuN* + neurons after transplantation of neural progenitor cells (NPCs) from embryonic day 14 *GFP* transgenic rats for 2 weeks. (**b**) Some grafted *GFP*-expressing cells also co-localize with the motor neuronal marker *ChAT*. (**c**) *GFP*+ axonal extension from graft into host. (**d**) *GFP*-labeled axons project into ventral roots and co-localize with the mature motor axonal marker *ChAT* in a confocal z-stack with xy, xz, and yz views). Scale bar: A, 128 μm; B, 28 μm; C, 64 μm; D, 16 μm

phenomenon in our study. Migrated human cells started around 3 months post-graft and expressed glial progenitor marker, vimentin. Human cells migrated from the graft site at a rate of 2–3 mm per month. They traveled and occupied only in host white matter (Fig. 6.3a, b). At 1 year post-graft, most migrated human cells express mature astrocyte marker, GFAP (Fig. 6.3c). Their processes were closely associated with host neurons and endothelium cells in the blood vessels. These results indicate that human astrocytes can migrate and spread into spinal cord to replace host astrocytes, which could directly protect motor neurons to prevent or slow down their degeneration in ALS.

9 Future Perspective

The therapeutic application of stem cells, including non-neural stem cells and neural stem cells (NSCs) and their derived progenitors for treatment of ALS, have great potential since stem cells provide neuroprotective effects and could replace degenerated motor neurons and supporting glia. The latter is especially true for NSCs and their derived progenitors, including motor neuronal progenitors and glial restricted precursors. In addition, there are varieties of resources of stem cells, especially for NSCs and their progenitors that can be generated from fetal

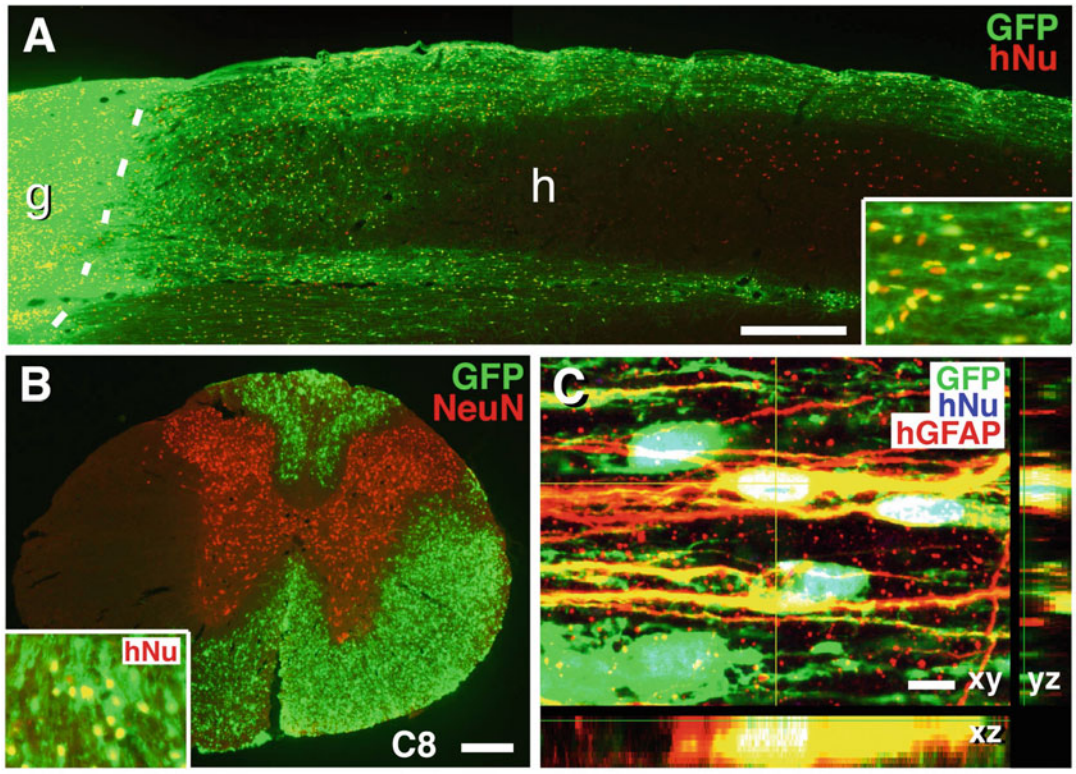

Fig. 6.3 Glial migration from human NSC graft into rodent host spinal cord. (**a**) Double labeling of *GFP* and human-specific nucleus (*hNu*) in a horizontal section reveals migration of human cells labeled by both *GFP* and *hNu* from NSC graft (g) into host (h) spinal cord (caudal shown) 18 months post-graft of human NSCs into C5 hemisection of immunodeficient rats. Dashed lines indicate graft/host interface. Inset shows co-localization of *GFP* and *hNu*. (**b**) Migrated human cells labeled by both *GFP* and *hNu* in a C8 cross section, three segments and about 9 mm below human NSC graft. (**c**) A Z-stack image showing co-localization of *GFP*+ and *hNu*+ human cells with human-specific astroglial marker *hGFAP*, in a horizontal section view at 18 months post-grafting. Scale bar = 600 μm (**a**), 280 μm (**b**), 7 μm (**c**)

CNS, ESCs, iPSCs, or direct conversion from adult somatic cells.

Despite there is great progress made in recent years for stem cell therapy in ALS, there is still a lack of consistent and meaningful improvement in disease progression and duration in both animal models and clinical trials. There are several challenges for stem cell transplantation therapy for ALS.

First, we do not know which stem cell type may offer the most therapeutic effect. While non-neural stem cells, such as MSCs, can modify the disease environment and may prevent motor neuron degeneration, only NSCs and their progenitors, such as motor neuronal progenitors, can replace degenerated motor neurons. Apparently, the naïve MSCs have only limited effect on the modification of disease environment. Genetic modification of non-neural stem cells to overexpress therapeutic genes, such as neurotrophins, may enhance their neuroprotection effect. Although NSCs can differentiate into neurons and glia, only motor neurons are appropriate neurons that need to be replaced in ALS. The transplantation of enriched motor neuronal progenitors could have a profound therapeutic effect. Functional motor neurons not only require their motor axon extending into ventral root and

peripheral nerve for neuro-muscular junction, but also receive appropriate supraspinal and pre-motor interneuron synaptic connection. The replacement of toxic glia, such as astrocytes, with health glial progenitor cells, along with motor neuronal replacement, could be another effective treatment.

The second challenge is where and how to deliver the stem cells in ALS patients. Since motor neuron degeneration is widespread in CNS, including spinal cord and brain, only global delivery, such as intrathecal injection of stem cells (Hwang et al. 2009), can reach the most affected CNS area. However, most of the injected cells stay at the meninges of the spinal cord and only a limited number of cells may migrate into spinal cord. In addition, migrated stem cells may randomly distribute in the CNS, most of which are away from degenerated motor neurons. On the other hand, intraparenchymal injection of stem cells has its advantage by placing the cells close to their therapeutic target, such as close to ventral horn of spinal cord. Several studies demonstrate that injection to multiple points to target both cervical and lumbar spinal cords is achievable in both ALS models (Xu et al. 2011) and clinical trials (Riley et al. 2012; Feldman et al. 2014; Glass et al. 2016). Nevertheless, intraparenchymal injections can only cover certain critical regions, but not all affected regions. The development of global delivery system that can effectively target degenerated motor neuron region is critical for the successful treatment of ALS.

Third, transplanted stem cells must survive in order to either deliver therapeutic molecules for neuroprotection or differentiate into neurons and glia for degenerated motor neuronal replacement and support. There are at least two factors that affect the survival of transplanted stem cells. The first one is the toxic environment of CNS that may affect survival of transplanted stem cells. Recently, our group developed a novel method to enhance grafted NSC survival in spinal cord injury models by embedding NSCs in fibrin matrixes that contain cocktails of 10 growth factors, which dramatically support the survival of grafted NSCs in the injury environment of spinal cord (Lu et al. 2012, 2014b). A similar method could be used to enhance the survival of transplanted stem cells in ALS models and clinical trials. The second factor is immune rejection of allogenic graft and an effective immunosuppressant or in combination could greatly enhance the survival of grafted cells (Yan et al. 2006). Unfortunately, none did a long-term study of immune-suppression on the survival of grafted stem cells in ALS models. Although iPSC-derived NSCs could be transplanted autologous without immune-suppression, iPSCs from ALS patients may have the same toxic genes that prevent autologous transplantation. Genomic editing using CRISPR/Cas9 could transform iPSCs derived from ALS patients into health cells, which could become a usable source of stem cells for autologous transplantation.

Fourth, transplanted stem cells may have a risk of safety issue, such as tumor formation. This is especially true for ESC- and iPSC-derived NSCs due to contamination of the original pluripotent stem cells that could form teratoma. In addition, the re-programmed iPSCs are from adult somatic cells that bear more risk genetics instability and tumor formation (Koyanagi-Aoi et al. 2013). An efficient method that can prevent tumor formation while preserving grafted neural cells, especially neurons, is needed for safe use of stem cells for the treatment of ALS and other neurological diseases and disorders.

Acknowledgments This work was funded by grants from the Veterans Administration (I01 RX002264-01A2) and NIH (R21 NS103074-01).

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Stem Cells Therapy for Multiple Sclerosis 7

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1 Overview of Multiple Sclerosis

Multiple sclerosis (MS) is the most common form of demyelinating diseases. It manifests with different symptoms in the areas it attacks in the central nervous system. Multiple sclerosis has shown to be a worldwide problem, with an approximate prevalence of 90 per 100,000 in the population, occurring at almost any age above 10, and causing different life limiting and debilitating states occasionally. The clinical management of MS costs billions of dollars yearly, and the number of patients with MS is estimated to be around 2.5 million worldwide. The patients mainly have African and Caucasian origins. There is no known cure currently available, but some proposed therapies exit aiming to inhibit the aggression of MS.

Many studies have shown that MS is the autoimmune disease "in progression" that involves the cellular apoptosis of nervous system and its components, the myelin. MS starts with damage from the myelin sheath of the central axons. The disease can start practically at any age, leading in its advanced form to an axonal degeneration. The disease prevalence has been on the rise in several areas around the world (Compston and Coles 2008).

However, most of the immune suppressive measures seems to be failed. Some medications are probably related to the immune modulatory potential of these agents, and this is shown with interferon beta family of drugs.

Other drugs affect the lymphocytes behavior as well. Their appearance at the global market was a total breakthrough as they are in oral form, but the usage of these oral agents is still limited and considered by Food and Drug Administration (FDA) as a second-line treatment due to their side effects on the cardiac, respiratory, hepatic, and ophthalmologic systems.

Probably, the monoclonal antibodies are the most clinically effective substances. That is what statistics showed in terms of reduction of clinical relapses (67%) and in terms of inhibition of imaginary progression (83%). But on the other hand, hives, pruritus, and the possible "anaphylactoid" infusion reactions are not the only adverse side effects; the concern goes into the direction of serious progressive multifocal leukoencephalopathy,

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F. Han, P. Lu (eds.), *Stem Cell-based Therapy for Neurodegenerative Diseases*, Advances in Experimental Medicine and Biology 1266, https://doi.org/10.1007/978-981-15-4370-8_7

making this therapy a somewhat of risky measure too.

It is important to mention that in the past chemotherapy was also used, and it was temporarily approved for treating this disease. It was considered as a last resort. All in all, these expensive drugs imposed heavy costs over the private sector and the assuring payer parties.

Based on the presence of a therapeutic gap in the treatment of MS, taking into consideration the final forms of disabilities we are still facing in our days, clinicians looked for a new, efficient approach for this devastating disease, and beside all the above named therapies, stem cell transplant for MS was proposed as an innovative therapy since the first decade of the twenty-first century.

2 Histopathology and Pathophysiology of MS

At the histopathological level of MS, it is noticed that the lesions are composed of areas of myelin and oligodendrocytes loss along with infiltrates of inflammatory cells, which include different lymphocytes and macrophages (Hauser 2008).

At the cellular level, oligodendrocytic progenitor cells, which are responsible for maturation as myelin-producing cells, regeneration, and continuous support, are aggressively attacked by the autoimmune system, leading to degradation and thinning of the myelin sheath. This destruction gradually leads to a clear electrical instability, weak axonal conduction, and a series of functional irregularities along the whole neuron, and not only the axon (Navikas and Link 1996). This pathological process which starts with inflammation passes through axonal degradation and dysfunction, leading to a glial scar formation, also named "sclerosis" (Holley et al. 2003).

The effector cells in MS include categories of T-lymphocytes, B-lymphocytes, natural killer cells, and oligodendrocytes. Deregulated immune inflammatory mediators include interferon gamma (IFN-γ), tumor necrosis factor (TNF), and several interleukins. This unusual combination of abnormal acting cells and factors interacts in a very complex schema to produce the clinical manifestations of MS (Calabresi 2004).

At the pathophysiological level, the degradation of myelin and what follows of astrocytic scaring in the tract of the postinflammatory healing process is behind the disruption of the electrical communication, resulting in a panoramic clinical tableau of a wide range of symptoms.

The diagnosis requires evidence of the disease activity on sequential examinations on the central nervous system magnetic resonance imaging. The incidence rises strongly between 20 and 45 years of age. The etiology and triggers of the disease remain poorly defined. Genetic and viral factors, along with environmental factors, are possible causes behind the etiology of MS (Barkhof et al. 2003).

Depending on three parameters – location, frequency, and degree of damage – lesions show up along the white matter and its tracts in the central nervous system, along the basal and lateral ganglia, the brain stem, the spinal cord, and the optic nerves. Symptoms correlate with the damage at the level of corresponding neurons. Statistically, the most known starting symptoms are weakness and tingling at random sites of the body, low visual acuity, nystagmus, and diplopia. Muscle spasms, chronic pain, dizziness, headaches, dysarthria, dysphagia, and urinary and bowel incontinence are some of those manifestations (Calabresi 2004).

The patterns and severity of symptoms are grouped into four subtypes (Lublin and Reingold 1996):

(a) *The Relapsing Remitting Multiple Sclerosis* (RRMS): This is the most popular type, which affects almost 80% of the patients, with a course of flareups alternating with periods of improvements. The symptoms are dynamic and abrupt with different degrees of recovery and periods of stability or slow progression.

(b) *Primary Progressive Multiple Sclerosis* (PPMS): Its course is usually progressive despite all measures, even the pharmacological treatment. This type affects about 10% of the patients.

(c) *The Secondary progressive Multiple Sclerosis* (SPMS): This type has a progressive course of symptoms after an initial course of a relapsing–remitting pattern, with or without periods of regression or stability.
(d) *Progressive Relapsing Multiple Sclerosis* (PRMS): It is the rarest type and is presented as an aggressive disease that continuously progresses and worsens.

Medicine is constantly searching for a curative treatment of MS, to everlastingly treat refractory cases which do not respond to any therapy. Known treatments of multiple sclerosis can be effective at relieving symptoms and spells but have no effect on the materialized demyelination, and there is no available cure.

3 Known Pharmacological Treatments and Neuroregeneration for MS

Pharmacological treatments nowadays introduce some immune suppression in a way to decrease the severity of the disease and in a best case-scenario to maintain it in a plateau state. No curative, FDA-approved therapies for MS are currently registered (Goldenberg 2012), and the first line of consensual treatment is known to be corticosteroids (Castro-Borrero et al. 2012).

Interferons, natalizumab, fingolimod, or glatiramer acetate may inhibit one of the multiple sclerosis's aggressive behavior found in tract of this disease, rather than restore any of its arms. The normal immune balance that explains the lack of full remissions or cure with all these medications carry the price tags of several thousands of dollars yearly (Coles Alasdair 2008).

Other current MS treatments are based on interfering with the leukocyte-specific adhesion molecules, helping to produce IL-4, IL-10, and TGF-beta, which are considered as immune suppressors. The same treatment may also inhibit the IL-2 and block CD20 and CD52 molecules, which have been shown to control the course of MS, but all for a limited a period of time (Corboy and Miravalle 2010).

A nervous system "injury" is not a term that encompasses all neuropathologies. Underdevelopment and/or biochemical, anatomical, and electrical malfunctioning can make the image more complete. Whether it is the brain, the cord, or the nerve, whether it is congenital or acquired, acute, or chronic, neural tissues take only two main kinds of etiologies of pathologies:

- Functional (infection/ischemia/inflammation/immune/insufficiency/intoxication) and
- Structural (accumulation/biodegradation/compression/disruption)

Neurogenetic could be a third independent category of neuropathological etiologies. Many of these causes are potentially an extension of other causes. Moreover, in their natural history, neurological disorders are presented with different timetable forms. They can have forms of attacks, cycles, episodes, be continuous, or have an irregular presentation. There is no strict time frame between different incidences and can sometimes jump from one frequency to another. We illustrate the gross time framing of these manifestations. And even with the advances seen at all specialty levels, they are the proposed cures in neuropathology that seem to be kind of shy. We can practically name no one disease that was cured, except for some infectious states. Therefore, the continuous research in this field attracted much support and financial coverage.

The idea of regeneration in the nervous system came from the acceptance of the fact that the recovery from a neurological disease is a very difficult matter of low hope. The degeneration happening at the end-stage of both structural and functional etiologies led the scientists to the stem cell road.

The available treatments for MS aim at providing partial relief of some symptoms, with no or little effect on the multifaceted pathology. There is no available cure, but rather soothing treatments to maintain the best available state for as long as possible. As successive preliminary clinical data on intravenous stem cells emerged,

multiple questions arose with regards to optimal methods for cell-based therapy in tract of MS treatment.

In 2009, under the title "Stem Cells Going Home," the Harvard Stem Cell Institute and the Harvard Department of Stem Cell and Regenerative Biology deliberated about signal sensitivity: In order to be successful in stem cell therapy and tissue regeneration, it is essential to increase the efficiency of stem cell homing, which is not easy to accomplish. A series of coordinated interactions between the stem cells and their environment provide the signals and signposts that guide them to navigate back to their niche or to be recruited by injured tissues. Meeting the challenges of understanding these complex mechanisms has a therapeutic potential in the field of stem cell biology.

The efficacy and specificity of the organ-specific progenitor stem cells proved its superiority in the clinical trials over the blank mesenchymal stem cells.

4 Stem Cell Therapy in the Treatment of MS

Beginning in 1995, Burt and his colleagues (1995) collected data from three separate institutions using stem cells in animal models with MS, and roughly concluded that the risk-to-benefit ratio of bone marrow transplantation (BMT) in progressive versus malignant MS cases is adequate for BMT in indolent lymphomas versus chronic-phase chronic myeloid leukemia (CML) cases (Table 7.1).

Five years later, when MS began showing strong circumstantial evidence that it is an autoimmune disease, Mandalfino et al. (2000) went into an experiment, treating an MS patient using his unaffected identical twin as a donor. This was because BMT was known to be a radical therapy for patients with life-threatening malignancies with a potential of treatment for human autoimmunity. Later on, they reported in their article "Bone Marrow Transplantation in Multiple Sclerosis" four cases of BMT, in which three included comorbid malignancies, and resulted in limited outcomes supporting further experimentation into this treatment modality.

During the same year, in 2000, based on the good results of experimental transplantation in animal models of multiple sclerosis and of other autoimmune diseases, Fassas et al. (2000) in Thessaloniki, Greece, treated 24 patients suffering from different primary and secondary progressive MS with a high dose of chemotherapy, followed by peripheral blood-derived autologous stem cell transplantation (the cytapheresis mobilization technique). This constituted a novel treatment with a specific therapy protocol. The group of scientists followed up their patients at a duration ranging from 21 to 51 months. The results were positive in 75% (18 of 24), where 9 patients out of 18 improved, and the other 9 developed relapses or they slowly resumed progression, keeping a stable disability after transplantation.

Saccardi and his colleagues retrospectively reviewed in 2006 a series of hematopoietic stem cell transplantations (HSCT) and 178 progressive cases of MS completed between 1995 and 2000. The overall mortality was 5.3% and that was probably related to the use of aggressive immunosuppression chemotherapy. Toxicity was registered as well and carmustine, etoposide, cytosine arabinoside, melphalan/antithymocyte globulin protocol was mainly used. Improvement or stabilization of neurological conditions occurred in 63% of patients at a median follow-up of 41.7 months, and HSCT was shown as a promising procedure to slow down progression of progressive MS.

In 2006, Lindvall and Kokaia showed that neurons and glial cells have been successfully cultured and neuro-endogenous stem cells immortalization trials were taken further. They were fully aware that the next step is a translation of these exciting advances from in vitro to clinically useful therapies.

In 2008, Karussis et al. wrote an article about the immunomodulatory and neuroprotective effects of mesenchymal stem cells, which were at that time named the bone marrow–derived non-hematopoietic or stromal cells. They have

Table 7.1 The non-hematopoietic bone marrow cells

Versatile non-hematopoietic stem cells described in BM	
Stem cells	*Phenotype*
MSC−[a]	International Society for Cellular Therapy criteria: CD105+, CD73+, CD90+, CD45−, CD34−, CD14−, CDllb−, CD79a−, CD19−, HLA-DR-
	Other additional markers: Stro-1+, SB-10+ (CD166), SH-2+ (epitope on CD105), SH-3+ (epitope on CD73), SH-4+ (epitope on CD73), CD44+, CD29+, CD31−, vWF−
	Markers of most primitive MSC: CXCR4, CD133, CD34, p75LNGFR
MAPC−[a]	SSEA-1+, CD13+, Flk-1low Thy-1low; CD34−, CD44−, CD45−, CD117 (c-kit)–MHC I-, MHC II-
MIAMI cells−[a]	CD29+, CD63+, CD81+, CD122+, CD164+, c-met+, BMPR1B+, NTRK3+, CD34−, CD36−, CD45−, CD117 (c-kit)-, HLA-DR-
MACS−[a]	CD13+, CD49b+, CD90+, CD73+, CD44+, CD29+, CD49a+, CD105+, MHC I+, HLA-DR−, CD14−, CD34−, CD45−, CD38−, CD133−, c-kit (CD117)-
VSEL stem cells	CXCR4+, AC133+, CD34+, SSEA-1+ (mouse), SSEA-4+ (human), AP+, c-met+, LIF-R- CD45−, Lin-, HLA-DR-, MHC I-, CD90−, CD29−, CD105−

[a]Phenotype of expanded/cultured adherent cells
AP Fetal alkaline phosphatase, *BMPR1B* bone morphogenetic protein receptor 1B, *c-met* receptor for hepatocyte growth factor, *LIF-R* receptor for leukemia inhibitory factor, *NTRK3* neurotropic tyrosine kinase receptor 3, *vWF* von Willebrand factor
Adopted from Ratajczak et al. (2008)

mentioned that bone marrow mesenchymal stem cells cultured with fibroblast growth factor (FGF) and brain-derived neurotrophic factor (BDNF) differentiate into neuronal/glial lineage cells, with a predominance of cells expressing astrocytes' markers. They also showed in a preclinical study a potential for migration of these cells bone marrow–derived mesenchymal stem cells (BM-MSC) into inflamed central nervous system tissue and differentiation into cells expressing neuronal and glial cell markers. Such an approach, they add, "may provide a feasible and practical way for *in situ* immunomodulation, neuroprotection and possibly remyelination/regeneration in diseases like multiple sclerosis."

In the same year, the same team of scientists generated another paper, titled "The Potential Use of Adult Stem Cells for the Treatment of Multiple Sclerosis and Other Neurodegenerative Disorders" (Slavin et al. 2008). They stated that cell therapy stands out as the most rational approach for neuroregeneration. They claimed an investigation of feasibility and efficiency of autologous mesenchymal stromal cells after their intrathecal and intravenous injection, with the purpose to induce in situ immunomodulation and neuroprotection and possibly neurorestoration in patients suffering of MS and other neurodegenerative disorders. They repeatedly said that preclinical results suggest that bone marrow may provide a source of stem cells with a potential for migration into inflamed CNS and differentiate into cells expressing neuronal and glial cell markers.

In 2010, Karussis and his colleagues registered again some advances in the field of stem cells biology after labeling the marrow-derived mesenchymal stromal cells with superparamagnetic iron oxide ferumoxides – Feridex. They intrathecally injected nine patients and tracked the labeled cells, confirming that they crossed the blood–brain barrier after visualizing them in the occipital horns of the ventricles by MRI imaging, indicating the possible migration of ferumoxides-labeled cells in the meninges, subarachnoid space, and spinal cord.

Moreover, an immunological analysis at 24 h after MSC transplantation revealed an increase in the proportion of $CD4^+$ and $CD25^+$ regulatory T cells, a decrease in the proliferative responses of lymphocytes, and the expression of $CD40^+$, $CD83^+$, $CD86^+$, and HLA-DR on myeloid dendritic cells. Concerning the short-term complications, the authors noted that injected MS patients had some transient low-grade fever and headache. During the follow-up period, no major adverse effects were reported, and at the clinical level the mean of the EDSS score

improved from 6.7 to 5.9 during the first 6 months of observation.

The year 2010 was really the year of advances. Martino et al. provided an overview about the potential use of transplanted stem cells in the treatment of multiple sclerosis. Two types of cells were used in an experimental autoimmune encephalomyelitis – the CNS-derived neural precursor cells (NPCs) and the bone marrow–derived mesenchymal stem cells (MSCs) – injected intravenously and intrathecally.

Preliminary safety data concerning the autologous intrathecally injected MSCs in patients with progressive MS are shown. But they chiefly stressed over the immunomodulatory and neuroprotective influences of both types of stem cells once they were transplanted systemically. This feature was accomplished by releasing a plethora of factors directly or indirectly influencing the regenerative properties of intrinsic CNS precursor cells instead of a solitary achievement by cell replacement.

Safety and feasibility of intravenous autologous bone marrow cell therapy was assessed in a phase I study by Rice et al. in 2010 too. Enrolled relapsing–progressive MS patients did not undergo any immunosuppressive preconditioning. After a clinical disability rating scales reassessment, multimodal evoked potential recordings, and a magnetic resonance imaging studies, beneficial effects were noted, including neurophysiological improvement without significant changes over a post-therapy period of 3 months.

In the 2010, Yamout et al. published their results of intrathecal injection of ex vivo expanded autologous bone marrow–derived mesenchymal stem cells in patients with advanced MS. Assessment at 3–6 months revealed EDSS improvement in 5 patients out of 7, stabilization in 1 patient out of 7, and worsening in 1 patient out of 7. However, on MRI, in 5 patients out of 7 they noticed new or enlarging lesions and in 3 patients out of 7 the lesions were enhancing. At the conclusion of this pilot study, the authors said that early results show hints of clinical but not radiological efficacy and evidence of safety with no serious adverse events.

The experimental autoimmune encephalomyelitis of Martino's team, the model for multiple sclerosis, pushed many other authors to dip into the analysis of what really is happening after the transplantation of stem cells.

Uccelli, Laroni, and Freedman wrote in 2011 that a subset of adult progenitor cells is an effective therapy in preclinical animal models of neurological diseases. MSCs ameliorate clinical course and decreases demyelination, immune infiltrates, and axonal loss. Surprisingly, these effects do not require full engraftment, but rely on the capacity of these cells to inhibit pathogenic immune responses and release neuroprotective and pro-oligodendrogenic molecules favoring tissue repair.

A prospective phase II open-label, single-center study on high-dose immunosuppressive therapy with autologous hematopoietic stem cell transplantation (AHSCT) in 95 patients with different types of MS was done in 2008 by Shevchenko et al. The mean follow-up period was 46 months, and the overall clinical response in terms of disease improvement or stabilization was 80%. No active, new, or enlarging lesions showed on the MRI.

There has been a lot of data about stem cells but a lack of randomized studies. In 2012, Connick et al. administered intravenous autologous bone-marrow–derived mesenchymal stem cells for a series of MS patients from east Anglia and north London regions of the United Kingdom at a mean dose of 1.6×10^6 cells per kg to secondary progressive MS patients in an open-label study. They registered the improvement after this treatment. The studied parameters included the visual acuity, the visual evoked response latency, and the optic nerve area which showed an increase. These changes in visual function, visual evoked response amplitude, and optic nerve area were at the base of their conclusion that stem cells probably worked by neuroprotection, a general idea resulting from the above-mentioned stem cell potentials (Connick et al. 2012).

Cohen in 2013 reviewed some of the mesenchymal cells features and wrote that they are pluripotent non-hematopoietic precursor cells

that can be isolated from bone marrow and numerous other tissues, relatively easily culture-expanded to purity, and induced to differentiate into mesodermal derivatives. He also mentioned in his paper that they exhibit many phenotypic and functional similarities to pericytes. Their immunomodulatory, tissue protective, and repair-promoting (tissue-restorative) properties demonstrated both in vitro and in animal models make them an attractive potential therapeutic tool for MS and other conditions characterized by inflammation or tissue injury.

In 2014, written in "Randomized Placebo-Controlled Phase II Trial of Autologous Mesenchymal Stem Cells in Multiple Sclerosis," Llufriu et al. enrolled nine MS patients who were unresponsive to conventional therapy and intravenously administered either placebo or bone marrow–derived mesenchymal stem cells. They followed the patients for 6 months and then reversed the treatment, so that those who received placebo were given MSC and vice versa. They also followed them for another 6 months and concluded that bone marrow–derived mesenchymal stem cells are safe and may reduce inflammatory MRI parameters supporting their immunomodulatory properties.

Harris and Sadiq wrote in 2015 in an article titled "Stem Cell Therapy in Multiple Sclerosis: A Future Perspective" the probable causes of differences in intravenous MSC administrations. They wrote that researchers in the UK conducted an open-label phase IIa study, in which they enrolled secondary progressive MS patients with optic nerve damage. Showing significant improvement in some visual end points, the study provided proof-of-concept data that intravenous MSCs may affect disease progression. However, less evidence of therapeutic effects was noted in a similar study conducted in the USA.

Unsurprisingly, despite their good viability, newly defrosted MSCs showed impaired immunoregulatory functions. This could explain why early trials showed less clinical efficacy compared with the results achieved when using freshly harvested cells in preclinical models. The intrathecal MSC-NP trial efficacy outcomes will be of interest since fresh, not cryopreserved, cells are delivered.

They also mentioned that compared with other routes of administration, the intrathecal route maximizes the therapeutic potential for delivery into the central nervous system.

And at the end of their paper, authors stated that the sooner the cellular was administered, the better the results are because of the glial scarring are happening in the tract of the disease.

Meamar et al. provided lately in 2016 an overview of beneficial stem cell transplantations and stem cell–based therapies, including sources, route, timing of each stem cell group, and finally, an overview of the stem cell research in clinical trial stages.

Ayache and Chalah also discussed in a paper in 2016 the promising abilities of stem cells therapies in MS patients, inviting to a larger scale randomized and controlled studies.

In a 2016 issue, Pickrell and Robertson criticized under a title "Stem Cell Treatment for Multiple Sclerosis" a specific type of autologous hemopoietic stem cell transplantation followed by an immunoablation protocol including combinations of chemotherapy, monoclonal antibodies, and anti-thymocyte globulin. They claimed that these treatments were inefficient. In autologous HSCT authors found that it was not easy to form sham and blind treatment groups, especially with those patients who already started pharmacological conventional treatments.

Karnell et al., in 2017, in a phase II clinical trial, achieved a result of a 5-year disease-free follow-up post intense immunosuppressive therapy combined with autologous $CD34^+$ hematopoietic stem cell transplant in 69.2% of treated subjects. The success was at three levels: There was no evidence of relapses, no loss of neurological function, and no new lesions on MRI.

Cohen et al. in 2017 studied the intravenously injected culture-expanded cryopreserved mesenchymal stem cells involving RRMS and SPMS patients. As for the results, the authors could not reach a definitive answer about the efficacy of their treatment but yet concluded that autologous MSC transplantation in MS appears feasible, safe,

and well tolerated. Future trials to assess efficacy more definitively are warranted.

A total of 16 children with pediatric MS improved in Expanded Disability Status Scale (EDSS) score in a study accomplished by Muraro et al. in 2017. They provided standard autologous hematopoietic stem cell transplantation (aHSCT).

5 Mesenchymal Stem Cells and Their Characteristics in MS

Stem cells are known to have marvelous features like self-renewal potential, remodeling capacity, differentiation, immune modulation, neuroprotection, and immune restoration. Stem cell therapies have given the MS and neuroregenerative fields new perspectives, especially with the fact of regeneration and immunomodulation.

Applications of stem cell has taken a lead among all other cellular revelations. Yearly, many conferences and publications witness the birth of new methods.

How to transform mesenchymal "blank" stem cells into other types of organ-specific progenitor stem cells is the main fantasy of all researchers.

It was shown that the stem cell immune modulator feature makes them capable of breaking auto-aggression processes. Stem cells have several properties including their ability of self-renew, remodel, differentiate, immune modulate, and restore immune balance.

Mesenchymal stem cells, a part of the bone marrow mononuclear stem cell team, are known for the majority of these features in particular the immunomodulation ones, a property that may be of utmost importance (Payne et al. 2011).

Le Blanc et al. (2003) reported that mesenchymal stem cells do not have alloreactivity lymphocyte responses because of their immunomodulation properties. This research group investigated the immunological characteristics of mesenchymal stem cells after their differentiation into three lineages: bone, cartilage, and adipose tissues. Although the HLA class I expression increased 6 or 12 days after growth, the HLA class II expression could not be detected.

Le Blanc et al. (2003) concluded that no lymphocyte alloreactivity was observed using MSC grown in osteogenic, chondrogenic, or adipogenic medium as stimulator cells. This supports that MSC transplantation can be performed between HLA-incompatible individuals. Later, many tests have been conducted, and the efficacy of mesenchymal stem cells/marrow stromal cells was proven in preclinical studies for many disorders.

When transplanted into the brain, MSCs stimulate the endogenous growth of neurons, decrease apoptosis and free radicals levels, promote synapses in damaged neurons, and control inflammation, mainly through paracrine activity. Joyce et al. (2010) showed that MSCs produce trophic factors that stimulate the regeneration and survival of host neurons, thus promoting functional recovery. Formerly, 15 MS and 6 amyotrophic lateral sclerosis patients were enrolled in an experiment to assess for the feasibility, safety, and immunological effects of intrathecal and intravenous administration of 40–60-day cultured mesenchymal stem cells derived from autologous adult bone marrow.

In their work, Karussis et al. (2010) noted transient fever and headache but found no major side effects. During the first 6 months, the mean Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS) score was shown to be stable. As for the mean EDSS score, it showed an improvement from 6.7 to 5.9.

MSCs were noticed in the occipital horns of the ventricles using magnetic resonance imaging, which points out the possible migration of ferumoxide-labeled cells in the meninges, subarachnoid space, and spinal cord (labeling by Feridex, the superparamagnetic iron oxide ferumoxides). After running immunological tests, the CD4+ CD25+ T cells proportion was found to be increased while the lymphocytic

proliferative response, the expression of CD40+, CD83+, CD86+, and HLA-DR on myeloid dendritic cells were shown to be decreased at 24 h after the MSC transplantation.

Gao et al. (2016) concluded that mesenchymal stem cells have a high therapeutic potential and can thus revolutionize the pharmaceutical field. For a clearer picture of MSCs immune mechanisms, they encouraged improved cell sourcing. They also supported the execution of proliferation and differentiation protocols in cell culture. The authors spoke about cryopreservation and quality control as well. They showed an optimistic view about the promising preconditioned and genetically modified mesenchymal stem cells and their potential therapeutic effects on immune diseases.

A large number promising MS treatments failed due to the complexity of the disease, which urges the researchers to search for more effective therapies directed to solve the immune damage and its consequent harmful effects.

Meamar et al. (2016) assessed the potential of the hematopoietic, mesenchymal, and neural stem cells in affecting the restoration of self-tolerance, immunomodulation, neuroprotection, and neuroregeneration. The efficacy and specificity of the organ progenitor stem cells proved its superiority in the clinical trials more than the blank mesenchymal stem cells.

The absolute number of stem cells and their different types needed for MS treatment are not known. Subjectively, I believe that the number and types of cells needed increase until the process of aging starts. The age at the start of the aging process cannot be exactly determined and it differs among individuals. It is practically approximated at the end of the anabolic phase of an organism. Moreover, it starts in each organ separately. Besides, there can be changes in the stem cell type, but my speculation will not be further explained in this chapter.

Unfortunately, we still find on the Internet pages some unreliable and invalid information like the one written in a post on a pathology student site, in which in the comment section about hematopathology (multipotent vs. pluripotent stem cells, 2013) the following is given: "Multipotent stem cells cannot give rise to any kind of cells in the body – they are restricted to a limited range of cell types . . . there are multipotent stem cells in the bone marrow that can give rise to red blood cells, white blood cells, and platelets. They cannot give rise to hepatocytes, or any other cell type, though – so they are not totipotent or pluripotent."

Huang et al. (2015) mentioned that "Molecular basis of embryonic stem cell self-renewal: from signaling pathways to pluripotency network" is one of the very few works that tackle the biomolecular basis of stem cell differentiation.

The work of Iain Murray and Bruno Péault, "Q&A: Mesenchymal stem cells—where do they come from and is it important?" which was published online on November 23, 2015, accurately defined mesenchymal stem cells and exposed many of their characteristics. Their work reviewed many of the stem cell features. Beginner researchers of stem cells therapy may undergo a short passage of nomenclature. Therefore, to enter this field, a clear-cut terminology differentiation is essential.

Minimally manipulated cells for homologous use (transplants or transfusions) are distinguished from cells regulated as medicines, as claims the EU regulatory classification of cell-based therapies. The cells that are adjusted as medicines have to meet some standard measures regarding their quality, safety, and efficacy in order to get a marketing authorization and then become commercially available (called Advanced Therapy Medicinal Products – ATMPs). As stated by cell-based therapy technology classifications and translational challenges (2015), they are also be subdivided into somatic cell, gene therapy, and tissue engineered products.

6 The Autologous Cell Transplant

Autologous stem cells were shown to be safe because of the full HLA and surface antigens compatibility and the possible rejections or unwanted reactions, as well no risk of genetic/DNA contamination. In 2015, 83,000 clinical trials were registered worldwide.

Some of these studies were using the allogeneic stem cells and others the autologous. There is no doubt that the use of embryonic stem cells leads to an ethical clash, which is related to the sacrifice of the embryos. In addition, any cells derived from embryonic stem cells are definitely allogeneic.

The exception is when the source of stem cells is the inner cell mass of a blastocyst or the placenta. The dilemma remains in the embryonic allogeneic cells, where a transfer of foreign genes from the donor to the hosting body stands as an additional risk of genetic material contamination, as long as the embryonic stem cells are from the same donor.

As a practical example of this therapy, an example is the use of the umbilical cord tissue–derived stem cells to treat cerebral palsy injury caused by perinatal lack of oxygen.

The Bone Marrow Transplantation There is no doubt that all the regenerative medicine pioneers were inspired by the bone marrow transplant works. The field of hematopoietic stem cells passed through a long and difficult way until it became after 30 years a well-formed arena. Together with the chemotherapy, they changed the destiny of many millions of blood disorder patients all over the world.

The Australian Centre for Blood Diseases at Monash University uncovered in January 2015 some additional amazing facts about the bone marrow in its publication named "The Red Cell Membrane": structure and pathologies – authors said that the blood vessels of the bone marrow constitute a barrier, inhibiting immature blood cells from leaving the marrow. Only mature blood cells contain the membrane proteins, such as aquaporin and glycophorin, that are required to attach to and pass the blood vessel endothelium.

It was proven that the bone marrow is in fact the mother place of many types of primitive stem cells, as given in "Bone Marrow – Home of Versatile Stem Cells," a publication that was chosen by many stem cell therapy pioneers to outsource their studies.

7 The Regentime Procedure

Due to the presence of a big gap in the treatment of MS and the major permanent disabilities that resulted, we (Regentime) felt the need to look for alternative ways and new approaches to treat this disease. To assess the patients, we used a validated assessment tool which is the EDSS score.

Score	Description
1.0	No disability, minimal signs in one functional system
1.5	No disability, minimal signs in more than one functional system
2.0	Minimal disability in one functional system
2.5	Mild disability in one functional system or minimal disability in two functional systems
3.0	Moderate disability in one functional system or mild disability in three or four functional systems. No impairment to walking
3.5	Moderate disability in one functional system and more than minimal disability in several others. No impairment to walking
4.0	Significant disability but self-sufficient and up and about some 12 h a day. Able to walk without aid or rest for 500 m
4.5	Significant disability but up and about much of the day, able to work a full day, may otherwise have some limitation of full activity or require minimal assistance. Able to walk without aid or rest for 300 m
5.0	Disability severe enough to impair full daily activities and ability to work a full day without special provisions. Able to walk without aid or rest for 200 m
5.5	Disability severe enough to preclude full daily activities. Able to walk without aid or rest for 100 m
6.0	Advanced disability where the patient requires a walking aid – cane, crutch,

(continued)

Score	Description
	etc. – to walk about 100 m with or without resting
6.5	Advanced disability where the patient requires two walking aids – pair of canes, crutches, etc. – to walk about 20 m without resting
7.0	Advanced disability where the patient is unable to walk beyond approximately 5 m even with aid. Essentially restricted to wheelchair; though wheels self in standard wheelchair and transfers alone. Up and about in wheelchair some 12 h a day
7.5	Advanced disability where the patient is unable to take more than a few steps. Restricted to wheelchair and may need aid in transferring. Can wheel self but cannot carry on in standard wheelchair for a full day and may require a motorized wheelchair
8.0	Advanced disability where the patient is essentially restricted to bed or chair or pushed in wheelchair. May be out of bed itself much of the day. Retains many self-care functions. Generally has effective use of arms
8.5	Advanced disability where the patient is essentially restricted to bed much of day. Has some effective use of arms retains some self-care functions
9.0	Advanced disability where the patient is confined to bed. *Still can* communicate and eat
9.5	Advanced disability where the patient is confined to bed and totally dependent. Unable to communicate effectively or eat/swallow
10.0	Death due to MS

Our study aim is to realize the proof of a concept using empowered autologous BM-MNSC, after their in vivo proliferation, to increase their number, and their short incubation in vitro to enhance their differentiation, all in order to demonstrate this method feasibility, safety, and efficacy in the treatment of MS.

7.1 Materials

We were interested in testing changes in the EDSS score after an autologous type of bone marrow mononuclear stem cells transplant along with a study of the safety and efficacy of this therapy. After the transplant, we concluded that the treatment was well tolerated and showed safety in long-term follow-ups. Moreover, an improvement at the EDSS score showed a decrease of 1 point, which was totally compatible with the global median results presented in similar studies using different types and methods of stem cell transplantation. Granulocyte colony-stimulating factor (G-CSF), the bone marrow mononuclear stem cells (BM-MNSC), and ACE are the three products used in our study.

G-CSF is the FDA-approved drug Filgrastim, the recombinant methionyl human granulocyte colony-stimulating factor (also abbreviated as r-metHuG-CSF) which serves to increase the number of the mononuclear cells in the bone marrow. The optimal dose of Filgrastim is 12–16 micrograms to each kilogram body weight per day for 2 consecutive days. Doses can be divided in two equal doses or injected as a single dose. The bone marrow is aspirated and collected from the patients themselves.

BM-MNSCs are autologous and extracted from an adult individual. They are first aspirated from the patient's bone marrow and meant to treat him/her and only him/her. They are separated and filtrated from the bone marrow aspirate using density gradient centrifugation before counting the cells using a Neubauer chamber. We did not use Sepax technologies, CHA Station, or any density gradient medium or plaque device. We did not store as well cells in any storing medium.

ACE is a sheep cellular extract designed for oral supplementation, produced by ACE Cells Lab Limited, United Kingdom. It is a peptide-length product made from live disintegrated cells of specific organs. It is manufactured in pico size, using advanced sonifying techniques and filtered to 300 kilo Dalton under strict EU specifications. Bio-farm closed-colony juvenile sheep are sourcing all types of ACE products. Some more available information about ACE

production (Xeno Organ Specific Eco Ultra Filtrates) is as follows: Under the UK laws, a bio-farm should substitute the laboratory with a new generation of juvenile sheep derived from a line where no registration of any single case of infection, genetic disorder, cancer of any type, or any transmitted and non-transmitted disease in at least last 20 generations. The placenta examination after each birth is checked, and an archived file is kept available till the sacrifice of the animal. Cellular extracts are manufactured as biological oral supplements for different human regeneration purposes. Both local and governmental veterinary regular control checkups are accomplished for each animal. A certificate of clearance is given. The producing laboratory is good manufacturing practice (GMP), good laboratory practice (GLP) certified, and the final product is checked by an independent third party.

7.2 Study on MS Patients

Between 2012 and 2015, 24 sequential patients with MS, 10 males and 14 females, aged 22–56 years old were accrued in this study using autologous bone marrow mononuclear cells. Seven patients were classified as having the SPMS type, eight as PPMS, and nine as RRMS. Pretreatment EDSS scores ranged between 1 and 8.5. All limbs of the study were reviewed and approved by the scientific committee and the Institutional Review Board at the hospital. The patients had to have confirmed MS disease by two independent neurologists from two different institutions, based on the clinical picture, the MRI, and the follow-up for more than single clinic visit. The MRI showing progression on two different occasions and the presence of deterioration over time were the chief conditions of patients' qualification to the study. All the patients also had a progressive course of the disease despite treatment with two or more standard lines of therapy, with the last medication used for more than 6 months. They also had normal cardiac, pulmonary, hepatic, and renal functions. All the patients signed an informed consent and received along with their families full explanation of the procedure, the protocol, the expected outcomes, and the eventual adverse effects.

7.3 Study Method

To quantify, monitor, and evaluate our patients before and after receiving this empowered BM-MNCS therapy, we used the EDSS, which is a validated reproducible tool that quantifies disability in eight functional systems and allows to quantitatively assign a functional system score in each case (Mezey et al. 2003). The protocol of the study has five stages: the pre-lab stage, the bone marrow collection stage, the laboratory stage, the transplantation stage, and the post-transplantation stage.

The Pre-Lab Stage Following basic laboratory tests, including coagulation study, radiological examinations, cardiologic, and anesthesia clearance, patients were qualified for the study, and G-CSF (Filgrastim) was subcutaneously injected in its approved dosage (10–15 μg/kg body weight) for 2 consecutive days. Patients were followed for eventual mid-low back pain, general bone pains, and abdominal fullness. White blood cell was counted twice: before the first dose of G-CSF and 6 h after the second dose. If adequate elevation in the peripheral white cell count was noticed, the patient was qualified for the next stage of bone marrow collection. Two patients from 30 had inefficiently increased the white blood cell count peripherally (named slow responders) and necessitated one to two additional doses, which meant longer preparation for one more day.

The Bone Marrow Collection Stage This stage started with a 6 h fasting, intravenous hydration, as a preparation for a smooth bone marrow collection procedure. All patients received sedation. After the patient is transferred to the clean operation room (with HEPA filter), he/she gets sedated

in a lateral position by the anesthesiologist. The area of bone marrow puncture was adequately scrubbed and draped.

The Puncture The iliac bone puncture can be done after accurate topographic localization and palpation of the posterior superior iliac spine. The puncture is done using a T-shaped biopsy needle – Jamshidi's type gauge 8, of 2–3 mm in diameter.

Two different directions for the puncture are taken – cephalic and caudal – reaching the cancellous bone of the pelvis bilaterally.

The aspiration of bone marrow happens in a speed of 1–3 mL/second after connecting the needle to a heparinized syringe.

The heparin dose is 5000 IU per mL in a volume of 10% of the bone marrow aspirate; 3 mL per 1 kg body weight of bone marrow extract was safely collected in a sterile way from the superior posterior iliac crest entry point. The extracted bone marrow is gently pushed into a single transfusion bag.

The laboratory stage includes the following steps:

(a) The first incubation:
The bone marrow underwent incubation at 20 °C for 24 h on soft mode shaker.

(b) Centrifugation and mononuclear cells collection:
A heavy-spin, low-speed centrifugation is typically accomplished to visualize a middle layer of the bone marrow – the cellular buffy coat including BM-MNSC.
The whole blood of bone marrow is separated into three layers from top to the bottom: plasma, buffy coat, and red blood cells. The buffy coat itself is composed of platelets and mononuclear cell layer (MNC), which contain stem cells and other cells including like lymphocytes, B-cells, T-cells, hematopoietic stem cells, MSCs, and dendritic cells.

(c) The second incubation:
The mononuclear cells were incubated again with a brain specific ACE (culture media manufactured by ACE Cells Lab Limited, Nottingham UK) for another 24 h at 20 °C.

The Transplantation Stage The final product was divided into two portions. The first portion was administered through an intrathecal injection at the lumbar area. This injection was directly preceded by cerebrospinal fluid drainage in a volume equal to the stem cells volume meant to be injected. For example, 15 mL of CSF was drained prior to a 15 mL injection of the cellular product. A bolus of 3 mL/kg body weight ml of 20% mannitol solution was given followed the lumbar puncture.

The second portion was given to the patient intravenously.

The Posttransplantation Stage This stage was equally important and based on the postoperative care needed to control eventual undesirable effects and transient symptoms like a headache or low-grade fever. All the patients were positioned in anti-Trendelenburg position for 1 h. Patients were discharged to go home 2 days after the procedure. They were initially contacted on a daily basis by the transplant team, and that was for the first week after discharge, then less frequent to a frequency of once weekly by the MS nurse, and monthly by us physicians.

7.4 Evidence of MS Improvement

Although the study was not placebo controlled, we showed improvement in graphical end points, providing proof-of-concept data that intravenous and intrathecal mononuclear autologous bone marrow–derived empowered stem cells may affect MS progression.

Therapeutic improvement was evident, and no similar study was performed on the universal level. We showed radiological improvement in the majority of functionally improving cases (Fig. 7.1).

In our study 19 of the 24 patients showed positive changes on EDSS score (79.1%). The improvement of 1 point on average on the EDSS

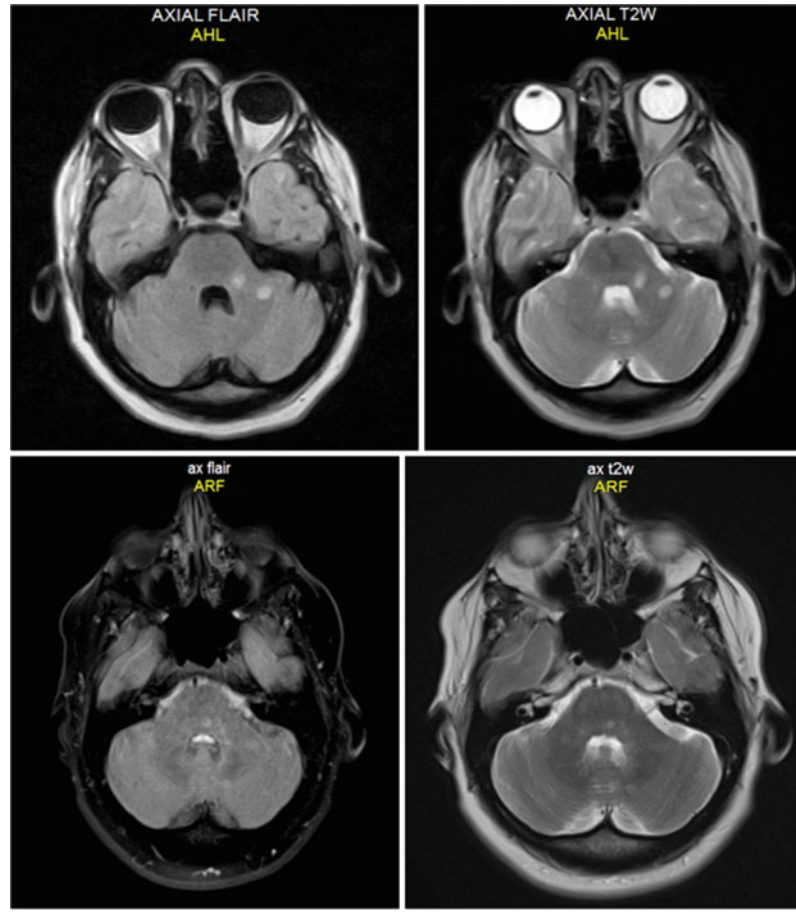

Fig. 7.1 MRI images of the cerebellum before and after the therapy: Top row: Two clear left-side cerebellar demyelination lesions before treatment with stem cell–based Regentime® procedure. Bottom row: Disappearance of the demyelinated cerebellar areas is noted after treatment

score was durable. Being able to ambulate independently or to live without the daily MS symptoms was significantly improving the quality of life and even drastically changing lives of some who regained their job, advanced in job position, married, and one patient got pregnant.

There were no long-term effects noted. There were also no issues to start the use of other therapies, and when comparing the EDSS scores before and after the injections, we found a trend toward improvement of the functional capacity, although the P-value did not reach statistical significance probably due to our sample size along with the study design as a phase II rather than a randomized trial.

The morphological changes denote that cells began transformation, and change in shape and pattern of the nuclei prove the transformation.

More studies are running to measure the physiological changes and viability in vivo, to detect all transformations, as well to measure all aspects of the clinical results using these reengineered progenitor stem cells.

7.5 Statistical Analysis

The procedure was well tolerated, with manageable pain at the site of bone marrow aspiration in 21 of 24 patients (87.5%), and its duration between 2 and 12 days (median duration 8 days). There were headaches in 15 of 24 patients

(62.5%), which lasted between a couple of hours and 3 days (median duration of headache 24 h). The third prominent adverse effect was the low-grade fever, which occurred in all the 24 patients (100%); nurses' charts registered an acute febrile state. One patient had nausea that lasted for 1.5 h and was easily controlled.

However, the expected EDSS scores to the posttreatment values, the calculated probability P-value, and statistical significance improved significantly to a two-tailed P-value of 0.04. We noticed that the progression is unpredictable and does not usually follow a clear pattern.

The 95% confidence interval was −1.003 to 2.653 with a mean of 5.3 before treatment and 4.475 after therapy.

The standard deviation widened from 2.5 to 3.2 posttreatment indicating the difference in responses seen among patients.

Even when the expected worsening is accounted for, the improvement showed a wide standard deviation again reflecting the variations among the patients' responses and some imaginary magnetic resonance studies show the areas of the demyelination process to have improved over time.

In our original study, we attempted to examine what may be the stem cells effect in their fortified form. We ran for G-CSF, the legendary stem cells mobilizer, in combination with incubation process. The transplantation routes were designed to be the least harmful for such category of patients, as long as there was no catheterization of the carotid arteries. The choice of routes was based on previous data and trials of others.

The novelty of our way depended on using complex in vivo and in vitro ways before in situ application on particular patients with MS.

We see the scientific revenue of our study interesting in the field of cellular biology and bioengineering as we observed the long-term safety of our therapy first. Responses were encouraging, even when sometimes varied between patients. The result was highly compatible with previous reports in the field (Abi Chahine et al. 2016a, b; Sharma 2002), although we noticed that the less the duration of the symptoms, the better is the response.

Our final injected product contains proliferated, partially differentiated human neural progenitor cells. These live cells, which can be kept in 4–8 °C temperature, hibernate for a safe duration of 2–3 days, and once they are injected into the host circulation, they travel to engraft probably by active homing into the meant organ, involving various chemokines including the stromal-derived factor (SDF-1). Opening of the blood–brain barrier using an intravenous bolus of 200 mL 20% mannitol solution facilitates the way to the progenitor cells to diffuse in, and undoubtedly allowing a higher concentration of neurotrophic factors to diffuse into the central nervous system issues, similar to the famous intrathecal chemotherapy technique.

The three registered posttransplantation symptoms – fever, nausea, and headache – were all due to one reason probably: To sterile meningitis caused by the rush of the cells, irritating the meninges.

Several growth factors like G-CSF, stem cell factor (SCF), the fibroblast growth factor (FGF), and others may help reduce the ischemia and enhance the migration and proliferation of the stem cells. There is evidence that the growth factors like the ones released from the stem cells may help improve neuronal regeneration (Klocke et al. 2008).

The transplanted bone marrow cells infiltrate the brain and may help regenerate new elements or combat the neurodegenerative process, fibrosis, and oxidative insults. Several studies reported significant improvement among patients with neurodegenerative conditions with no significant adverse events. In this study, we reported a significant improvement in 79% of the patients treated for progressive MS with minor adverse events and great tolerability as reported by other groups (Chaitanya et al. 2012); others used BM-MNSCs to treat cerebral palsy, giving five intrathecal injections, and reported major clinical improvement and high safety.

All these studies confirm the safety profile of MSCs, whereas the discrepancy in their outcomes

could have been derived from various points of difference that deserve to be addressed.

In 2017, stem cell laboratories are operational in more than 70 countries. We have in our disposal hundreds of precursor cell types and thousands of tissue culture media. Stem cells did not only transfigure the treatment of cancer, but they also founded regenerative medicine and fueled the emergence of a biomedical industry all over the world.

Bioengineering using stem cells achieved some advances using catalysts of growth factors. It succeeded in several applications to generate ectodermal organ–specific stem cells like neurons, mesodermal like keratinocytes, and osteocytes or endodermal organ specific cells like hepatocytes.

8 Conclusion

Stem cell field is revolutionizing the medicine, its expectations exceeded all past predictions. Bioengineering is becoming one of the most advanced techniques these days. It is dedicated to control stem cells. Since the first robust years of the twenty-first century, stem cell research was directed toward a clinical vision. The difference between one type and the other is the potency. Our cells can be combined with other therapeutic approaches and tested extensively in phase III studies to demonstrate and prove their efficacy beyond doubt. The injections can be repeated to sustain and to enhance the results. It is not clear at this time what is the best quantity or the best therapeutic schedule, or whether it is beneficial to add some more medications to maximize the stem cell effect in the course of MS management. In this study, we reviewed and followed the post-therapeutic state of 24 patients treated with bone marrow–derived empowered mononuclear stem cells. The cells were given using intravenous and intrathecal routes. Our list of patients adds to the current evidence that the BM-MNSCs are safe and partially effective in many patients with MS. A lot of work remains, but we have to elaborate more on this line of therapy requiring a clear and strict methodology to keep abiding by the medical ethics. The concept of cellular therapy entails a fundamental new vision at the pharmacodynamics and pharmacokinetics along with novel roles for using differentiation growth factors and special in vivo microenvironments reaching a redefinition of the phases of the clinical studies. Stem cell therapy may be the future pathway to solve a large number of debilitating and disabling multiple sclerosis cases, just if we can control who and how can channel the therapy into the correct form.

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Cell-Based Therapy for Spinal Muscular Atrophy

8

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1 Introduction

Spinal muscular atrophy (SMA) is a rare genetic disease with severe neurodegenerative consequences. Commonly SMA is a childhood autosomal recessive neuromuscular disease due to mutation in survival of motor neuron 1 (SMN1) locus responsible for ubiquitous SMN protein production (Lin et al. 2015). SMN is a 38 kD protein, ubiquitously expressed in different cell types. SMN was first reported in nucleus and cytoplasm in HeLa cell lines. Within nucleus SMN is more localized forming "Gems," or Gemini of coiled bodies (Cajal bodies), interacting with RNA-binding proteins. Reduced SMN protein is responsible for alpha motor neuron degeneration in the brain stem and in the ventral horn of the spinal cord, resulting in progressive skeletal muscle weakness and atrophy. As consequences, paralysis, brainstem (bulbar) defect, and respiratory defects are the early manifestations leading to shortened life span and death as final outcome (Sumner 2006). The incidence of SMA has been estimated approximately 1 in 10,000 newborns, representing the second most common neuromuscular disease with an expected carrier frequency of 1 in 40–60 (Pearn 1978; Prior et al. 2010).

2 Etiology and Molecular Genetics

SMA is caused mainly by homozygous deletion or mutation of the telomeric SMN1 gene, located on chromosome 5q11.2–q13.3 (Lefebvre et al. 1995). The centromeric SMN2 gene is a paralogue to SMN1, produced by intrachromosomal duplication of 5q13. Genetic linkage studies revealed that the SMN protein, the product of SMN1 and SMN2, is considered as disease causing agent in SMA. In fact, SMN2 gene differs from SMN1 by only a few nucleotides at base pair position 840, resulting C to T substitution in an exonic splicing enhancer site of exon 7. However, the base substitution is translationally silent but impairs the functionality of spliceosome to recognize exon 7. Eventually exon 7 is skipped from approximately 85–90% of mature mRNA transcripts, generating a truncated

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F. Han, P. Lu (eds.), *Stem Cell-based Therapy for Neurodegenerative Diseases*, Advances in Experimental Medicine and Biology 1266, https://doi.org/10.1007/978-981-15-4370-8_8

unstable protein (SMNΔ7) (Cartegni and Krainer 2002). However, SMN2 gene is retained in all SMA patients and produce low level (approximately 10%) full length of SMN2 protein by the alternative splicing, which cannot provide compensation for SMN1 mutation-caused deficiency (Nurputra et al. 2013; Lefebvre et al. 1995).

3 Clinical Classification and Pathological Mechanisms

Clinically SMA is presented as multiple phenotypes and severity of the disease is inversely associated with copy number of the SMN2 gene (Kolb and Kissel 2015). Based on the age of onset and the severity of the motor function, SMA can be classified into four groups: SMA Type 1 or Werdnig–Hoffmann disease (age of onset 0–6 months, never sit); SMA Type 2 or intermediate type (age of onset 7–18 months, sit but never stand); SMA Type 3 in adulthood or mild type/Kugelberg–Welander disease (age of onset >18 months, stand and walk during adulthood); and SMA Type 4 or adult type (age of onset second and third decade of life, walk unaided). Additionally, a specific SMA Type 0 has been reported with severe phenotype having prenatal onset (Kolb and Kissel 2011; Mercuri et al. 2018). The clinical classification of SMA is shown in Table 8.1. Clinical findings and molecular genetic testing are the gold standard in the diagnostic process of SMA. Clinically, infants present with hypotonic, progressive symmetric proximal weakness preferably in lower limbs, often with bulbar muscles but not in facial muscle. Intercostal muscles were involved in sparing diaphragm presenting "bell-shaped" chest with paradoxical breathing. Childhood onset predominantly presents with proximal muscles weakness (Mercuri et al. 2018). In a typical clinical presentation, muscle biopsy is not recommended; even electromyography (EMG) for type 1 and type 2 is not required. In few exceptional cases, serum creatine kinase (CK) may be highly elevated; however, CK level is not considered as diagnostic exclusion tool. For a clinically suspected case, genetic testing of SMN1 and SMN2 is regarded as highly reliable and first-line investigation. SMA genetic testing panel includes quantitative analysis of both SMN1 and SMN2 using multiplex ligation–dependent probe amplification (MLPA), quantitative polymerase chain reaction (qPCR), or next-generation sequencing (NGS). Since SMA is a multisystem disorder, multidisciplinary approach is the key element in the management of SMA patients. Considering motor neurons are the primary pathological target, development of SMA therapeutics has been revolutionized in recent years.

4 SMN-Based Gene Targeting Strategies

4.1 Protection of Motor Neurons

Numerous pharmacological agents have been used in various preclinical and clinical studies to evaluate their safety and benefit in providing neuroprotection for SMA. Riluzole and gabapentin could decrease the level of glutamate in presynaptic neurons in mice by minimizing glutamate excitotoxicity. But in another study, the placebo-controlled trials of gabapentin could not show significant benefit in patients with SMA type 1 and type2 (Merlini et al. 2003; Miller et al. 2001; Russman et al. 2003). Nizzardo et al. (2011) have shown that ceftriaxone, a β-lactam

Table 8.1 Clinical classification criteria for spinal muscular atrophy

SMA types	Age of onset	Highest function achieved
Type I (Werdnig–Hoffmann disease)	0–6 months	Never sit
Type II (intermediate)	7–18 months	Sit never stand
Type III (mild, Kugelberg–Welander disease) in adulthood	>18 months	Stand and walk during adulthood
Type IV (adult)	2–3° decade	Walk unaided

antibiotic, also provided neuroprotection in a mouse model. Ceftriaxone could increase the general weight, muscle size, motor neuron numbers, and neuromuscular junctions (NMJs) by increasing the level of glutamate transporter EAAT2/GLT-1, transcription factor Nrf2, and SMN. Thyrotropin-releasing hormone (TRH) showed some transient improvement by its trophic effect in SMA patients (Kato et al. 2009; Tzeng et al. 2000). Olesoxime, a cholesterol-like small molecule, has drawn much attention due to its neuroprotective properties. Preclinical study suggested that Olesoxime can prevent mitochondrial dysfunction in motor neurons and has therapeutic potential for SMA (Bordet et al. 2007). Bertini et al. (2017) published the result of the first phase II clinical trial with Olesoxime and placebo over a period of 24 months in patients with SMA type 2 and type 3. However, their primary end point was not met, but results suggested that Olesoxime might provide meaningful in clinical benefits for patients with SMA. Another open-label phase II study has been started in 2016 to evaluate long-term safety, tolerability, and effectiveness of Olesoxime in SMA patients, expected to be completed by May 2021 (Medicine 2015).

Insulin-like growth factor 1(IGF-1)–induced signaling networks play a vital role in modulating cellular process (i.e., cell growth, proliferation, and survival) (Vardatsikos et al. 2009). Tsai et al. (2012) demonstrated that CNS-directed IGF-1 administration could decrease motor neuron death in SMA mice (SMN−/−SMN2+/−). Murdocca et al. (2012) used recombinant human insulin-like growth factor 1 (rhIGF-1) combined with recombinant human IGF-1 binding protein 3 (rhIGFBP-3) in a preclinical study on mouse model and reported beneficial effect on the survival of motor neurons as was evident by reduction in motor neuron degeneration, increased muscle fiber size, and elevation of motor function of SMA mice.

4.2 Protection of Non-neuronal Tissues

Along with other supportive care, proper nutritional management is important in SMA patients. Butchbach et al. (2010a) showed that SMA mice fed with higher fat diet survived longer than those fed lower fat diet. Muscle mass improvement was reported following inhibition of myostatin by overexpression of follistatin and expression of IGF-1 (Bosch-Marce et al. 2011; Sumner et al. 2009). Nevertheless, none of these agents could ameliorate motor function but showed different positive effects in the muscles of SMA mice (Bosch-Marce et al. 2011; Murdocca et al. 2012).

Some evidence suggests that the RhoA/ROCK pathway has significant role in the SMA pathogenesis (Bowerman et al. 2009; Nölle et al. 2011). ROCK inhibitors (Y-27632 and Fasudil) can improve the survival of Smn2B/− mice, which may be due to improvement in NMJ maturation and increment of muscle fiber size (Bowerman et al. 2010, 2012). However, administration of Y-27632 was not effective with the most severe SMA phenotype in Smn−/− mouse model and suggested specific therapies (Bowerman et al. 2010).

4.3 Small Molecule drugs

Small molecule drugs have been investigated to modulate endogenous SMN2 and found to be effective in increasing SMN level in vivo. Among them histone deacetylase inhibitors (HDACis) are noteworthy. It is postulated that acylated histone allows active transcription of corresponding chromatin region. Some HDAC is (valproic acid, phenylbutyrate) have been shown to increase SMN level by increasing SMN2 expression by enhancing SMN 2 promoter activity. Only two of the HDAC inhibitors (4-phenylbutyric acid [PBA] and valproic acid [VPA]) have entered clinical trials. HDACis are not specific, produce off-target-related toxicities, and provide no or less therapeutic promise (Mohseni et al. 2013; Wadman et al. 2012).

Another FDA-approved small molecule drug aminoglycosides can elevate SMN level in the neurons, when administered directly to the CNS. Results showed that aminoglycodises can reduce the severity of SMA phenotype. However, this difference was not statistically significant (Mattis et al. 2009).

4.4 Gene Therapy

In recent years, Gene therapy has achieved some striking results both in preclinical and clinical research in the treatment of SMA. Research mainly emphasized on two strategies: (1) antisense oligonucleotide (ASO) to increase SMN protein production in SMA; (2) virus vector (scAAV9) to carry SMN gene to repair the defective SMN1. In the last decade, some research results have come from several early SMA patient trials (Fuller et al. 2010).

4.4.1 Antisense Oligonucleotides

Antisense oligonucleotides are synthetic, short (15–25 nucleotides), single-stranded DNA or RNA sequences, which have been recognized since 1970s for potential therapeutic use. Several decades of research in modification has made advancement in the treatment of SMA. The biochemical mechanism of mRNA splicing is highly complicated and depends on multiple levels of regulation through interaction between pre-mRNA, protein splicing factors, and small nuclear ribonucleoproteins. ASOs can hybridize to splice sites, silencer or enhancer elements, on the transcripts resulting in exon skipping, restoration of splicing pattern, or shifting the ratio between existing splicing forms, according to desired intention (Rinaldi and Wood 2018; Tosolini and Sleigh 2017). The intronic splicing silencer N1 (ISS-N1) which is a 15-bp nucleotide sequence found in intron 7 of SMN2 pre-mRNA has critical role in splicing regulation. A modified ASO, nusinersen, complementary to the ISS-N1 in intron 7 of SMN2 pre-mRNA is specifically targets SMN2 pre-mRNA (Singh et al. 2006). Specific ASO/pre-mRNA hybridization restricts exon 7 mis-splicing to increase the amount of functional SMN produced by SMN2 and results in improved survival and the motor phenotype in mouse models of SMA (Passini et al. 2011; Wood et al. 2017).

Nusinersen, a modified 20-O-2-methoxyethyl phosphorothioate antisense oligonucleotide to treat all subtypes of SMA patients, has already been approved by the FDA on December 2016. In vitro assays and studies in transgenic animal models of SMA showed nusinersen to increase exon 7 inclusion in SMN2 mRNA transcripts and produce full-length SMN protein. Furthermore the analysis of autopsy-derived thoracic spinal cord tissue preparations revealed that exon 7 was included in 50–69% of SMN2 mRNA transcripts from three infants with SMA who were exposed to nusinersen in the phase II trial (Study CS3A; NCT01839656) (Hoy 2017).

Despite the striking outcome of nusinersen, there are some questions to be addressed. Until now nusinersen was used in a clinical trial on patients less than 12 years old, hence long-term monitoring in adult patients is required (Parente and Corti 2018). Since SMN is a ubiquitously expressed protein, thus SMA is a general splicing disease that is not restricted to motor neurons. A planned interim analysis of ENDEAR trial revealed that intrathecal administration mainly targets the CNS, doesn't cover the systemic organs, and covers up only 40% patients (Stein and Castanotto 2017). Treatment of disease involving skeletal muscle, motor circuits, and multiple subpopulations of neurons, etc., might be an issue in future. Moreover, treatment with nusinersen is extremely expensive. According to dosage protocol, the treatment cost is approximately $750,000 in the first year and $375,000 annually in subsequent years besides other related medical costs. There are many practical challenges which has to be overcome in the future (Burgart et al. 2018).

4.4.2 scAAV9 as Gene Therapy

A second approach in gene therapy is to introduce SMN1 gene by using nonreplicating self-complementary adeno-associated virus serotype 9 (scAAV9). Mulcahy et al. (2014) has shown promising results in preclinical studies. Unlike nusinersen, AAV9 was shown to cross the blood–brain barrier in mice, cats, and nonhuman primates, permitting intravenous route of drug administration (Samaranch et al. 2011). Furthermore, in contrast to nusinersen, AAV9 due to its neuronal tropism properties, provides stable and long-term expression even with a single administration. Thus, AAV9 can minimize

immunogenicity issues associated with virus vectors (Lorain et al. 2008) and overcome the complications arisen from multiple, invasive intrathecal injections (Haché et al. 2016). An open-label, dose-escalation phase I clinical trial completed in a patient with SMA type I by AVXS-101 injected intravenously. The results of the trial showed that AVXS was safe, well tolerated, and resulted in longer survival, superior attainment of motor milestones, and motor function in comparison to historical SMA type I cohorts (Mendell et al. 2017). However, viral vector–derived immunogenicity issues still remain challenging.

4.5 Stem Cell Therapy

Stem cells are well known to have potential to replace and repair almost all types of tissues as well as neuronal tissues. Numerous preclinical studies showed that different types of stem cells have been used to protect and/or replace lost motor neurons, interneurons, as well as nonneuronal cells in neuro-muscular diseases. Along with other approved therapeutic options, cell therapy also makes contribution in helping to improve the clinical outcome of SMA, including embryonic stem cells. For example, embryonic stem cells can differentiate into neurons and form neuromuscular junctions to improve neuromuscular function. Induced pluripotent stem cells (iPSCs)-derived motoneuron in SMA murine model, sciatic nerve injured mouse model, and injured musculocutaneous rat models have shown their ability to recover neuromuscular function and delay the muscular atrophy. Furthermore, neural stem cells can reduce the neuronal death; differentiate into motor neurons having doublecortin, LIS1, and drebrin gene (gene of neural differentiation); and express the normal neural protein and neurotransmitter to restore the neuromuscular functions of SMA murine model (Corti et al. 2005, 2008; Pepper et al. 2017; Su et al. 2018). Recently, researchers established prenatal diagnosis of SMA as an outcome of genetic screening and provided prenatal cell therapy for SMA in mice in order to prevent irreversible damage that might occur in utero. Shao-Yu Peng (2018) injected human amniotic fluid stem cells (HAFSCs) in type 3 mice during second trimester of pregnancy. They reported that HAFSCs transplantation significantly improved motoneural function and survival in later life.

4.5.1 Mesenchymal Stem Cell (MSCs) in Spinal Muscular Atrophy

MSCs have been shown sparking promises in numerous preclinical and clinical studies in the treatment of different neurological diseases. Mesenchymal stem cells are well known for their immunomodulatory properties in critical environment. MSCs can neutralize neurotoxin and are able to provide neuroprotection by producing bioactive neurotrophic factors and thus stimulating local progenitor cells to replace SMN1 and eventually differentiate into functional neural cells (Murphy et al. 2013; Paradisi et al. 2014; Villanova and Bach 2015). Villanova et al. (2015) collected allogenic bone marrow mesenchymal stem cells, expanded in vitro, and transfused them in three children with spinal muscular atrophy type 1 intrathecally and intravenously. The MSC treatment was safe and showed quantifiable improvements in physical function at least during the treatment. They suggested to consider the outcomes as an initial step for a larger study.

4.5.2 iPSC in Spinal Muscular Atrophy

Global burden of genetic diseases is attributed to poor advancement of research due to a lack of reliable models. Possibly fibroblast cells from the SMA patient are the most studied cells in culture due to easy collection, easy expansion and maintenance in culture, and natural lack of SMN1. But unfortunately fibroblasts do not differentiate into motor neurons, astrocytes, or muscles, a big obstacle in SMA research (Van Damme et al. 2007). The concept and development strategy of patient-specific iPSC generation from SMA patients is believed to overcome the scarcity of motor neuron as a research model.

iPSC derived from the skin fibroblasts of SMA patients has been attracting growing interest due to not only its easy collection, but also unlimited production of physiologically relevant,

pathologically differentiated cells. Ebert et al. (2009) demonstrated the generation of iPSCs from SMA patients through viral vector approach from a skin fibroblast sample and subsequently differentiated into motor neurons.

Tammy Chang et al. (2011) showed that clonal variation of iPSC is not responsible for the reduced capacity to differentiate into motor neurons by the SMA iPSCs, rather it correlates with inherent genetic defect. It was evident by the wild-type SMN expression in these iPSC lines that could restore normal motor neuron differentiation. This study provided strong support for using the iPSC as human cell model of SMA. Heidi R. Fuller et al. demonstrated that human motoneurons differentiated from iPSC derived from type I SMA patients and showed neuron-specific consequences of SMN depletion in contrast to healthy control provides a concept for better understanding of molecular consequences and identification of therapeutic targets for SMA-derived (Fuller et al. 2015). Diederichs and Tuan (2014) MSC-like cells generated from iPSCs that could open the possibility to generate large amount of uniform batch of MSCs. Thus, iPSC-derived MSCs could be a novel source for SMA research leading to functional treatment (Diederichs and Tuan 2014). Mai Feng hypothesized that overexpression of SMN protein may produce a better therapeutic effect in comparison to MSCs with no transgenic SMN1. Consistently, they generated SMN1-MSCs by using human rDNA-targeting vector and TALENickases to targetSMN1 into SMA-iPSCs, with relative target efficiency up to 44.4%. PCR and Southern blotting analysis confirmed the site-specific incorporation of exogenous gene in SMA-iPSCs (Feng et al. 2018). Expectedly, the differentiated SMN1-MSCs produced increased number of SMN multiprotein complexes (gems), and an increase in SMN expression that is postulated to be associated to the disease severity (Arnold et al. 2002; DiDonato et al. 2003; Rashnonejad et al. 2016).

Stefania Corti et al. successfully generated iPSC from SMA (SMA-iPSCs) patients by non-viral and non-integrating episomal vectors with a view to correcting targeted single-stranded oligonucleotides that could convert the SMN2 gene into an SMN1-like gene. To determine whether mutation-corrected SMA-iPSC-derived motor neurons survive, engraft appropriately, and ameliorate the disease phenotype, human-derived GFP-labeled motoneurons were transplanted to the spinal cord of SMA transgenic mice. It was found that SMA-iPSC-derived motor neurons integrated within the ventral horn gray matter of the spinal cords of all transplanted animals. In addition, the lifespan of experimental animals and their disease phenotype were improved by the transplantation of derived motor neurons from SMA-iPSC than that of uncorrected SMA-iPSCs (Corti et al. 2012). This study provided solid evidence for the generation of genetically corrected iPSC for clinical translation in stem cell-based treatment for SMA.

4.6 Mutation correction by CRISPR Technology

Generation of iPSC by vector based approach might entangle into insertional mutations, transgene expression, and tumorigenesis. In order to solve this obstacle, the CRISPR/CRISPR-associated protein (Cas) system in gene engineering has been surprisingly harnessed a revolution. A large amount of studies revealed that CRISPR/Cas9 system is a promising gene-editing tool, which holds a clinical potential for curing SMA and other genetic diseases (Okita et al. 2007; Yu et al. 2007). Recently, CRISPR-Cpf1, as an RNA-guided, class II CRISPR/Cas system that is analogous to CRISPR-Cas9, showed its efficacy in precise double-stranded break (staggered manner) and reducing off-target effect in comparison to CRISPR/Cas9 (Kim et al. 2016; Kleinstiver et al. 2016; Yan et al. 2017; Zetsche et al. 2015).

Miaojin Zhou et al. used CRISPR/Cpf1 and single-stranded oligodeoxynucleotide (ssODN) to generate c-Myc-free, non-integrating iPSCs from urine cells of an SMA patient. This experiment showed that CRISPR/Cpf1 genome-editing system efficiently converted SMN2 to an SMN1-like gene in SMA patient that could restore SMN

expression in the iPSCs and differentiated motor neurons (Zhou et al. 2018).

The delivery route is clearly crucial to therapeutic success, including patient's compliance. Till now in different clinical trials, drug delivery to CNS had obtained better outcome despite risk and delivery-related hazards. Moreover, in long term clinical trials assessment of SMN protein level in the cells of SMA patients is necessary. SMN spot analysis in peripheral blood cell (PBCs) by flow cytometry may become primary endpoint assay for the evaluation and monitoring of SMA therapeutic intervention (Otsuki et al. 2018). However, until now no single treatment strategy has been found sufficient for SMA patients. Future endeavor with combined drug and delivery approach might provide a momentum in this field of research and development for inherited diseases.

Acknowledgment This work was supported by fund from Department of Science and Technology of Shandong Province, China to Dr. Fabin Han (2017GSF18104).

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Stem Cell Transplantation Therapy for Retinal Degenerative Diseases

9

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1 Introduction

Retinal degenerations such as age-related macular degeneration (AMD), macular dystrophy, and retinitis pigmentosa (RP) are the major causes of the aging blindness, because of the neurodegeneration of the photoreceptors and their supportive retinal pigment epithelium (RPE) in the retina. The most common retinal degenerative disease is AMD, of which about 80–90% are dry AMD caused by eventual degeneration of RPEs and photoreceptors. The other 10–20% are wet AMD caused by abnormal neovascularization and subsequent fibrosis in the retina (MacLaren and Pearson 2007). Another common retinal degeneration is Stargardt disease (STGD), which is an autosomal recessive macular dystrophy in that the affected patients have early disease onset in the age of about 5–15 years. Most patients with STGD are affected by genetic mutations in the *ABCA4* gene to accumulate the highly toxic A2E, leading to degeneration of RPE and subsequent dysfunction of photoreceptors. Mutations in *BEST1* cause retinal disorders of bestrophinopathies, which mostly affect the functions of neural circuit pathways in retina. The most common macular dystrophies of bestrophinopathies are macular dystrophy (BVMD) and autosomal recessive bestrophinopathy (ARB) (Guziewicz et al. 2017; Tanna et al. 2017). In these retinal degenerative diseases, initial RPE lesions eventually lead to subsequent degenerations of photoreceptors to cause blindness. Since retinal degenerative diseases are mainly caused by progressive loss of RPE cells, photoreceptors, ganglion cells, or microvascular cells (endothelial and pericytes) in the retina, stem cell replacement therapy will provide great perspective for patients with RDD (Li et al. 2016b).

The purpose of stem cell therapy for retinal degeneration is to use transplanted stem cells to replace the degenerated retinal cells in the retina for functional restoration of the vision. In the past decade, a lot of the research has extensively been conducted to replace degenerated neural cells in the retina with different stem cells such as hESCs, iPSCs, fetal neural stem cells, and adult bone marrow–derived mesenchymal stem cells (MSCs) (Ramsden et al. 2013). Because of their pluripotency, hESCs and iPSCs have been efficiently induced to generate functional RPE (hESC/iPSC-RPE) or photoreceptors in vitro.

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F. Han, P. Lu (eds.), *Stem Cell-based Therapy for Neurodegenerative Diseases*, Advances in Experimental Medicine and Biology 1266, https://doi.org/10.1007/978-981-15-4370-8_9

Many preclinical studies have differentiated both hESCs and iPSCs into retinal cells that display morphological and functional similarities with photoreceptors and RPE cells of the retina. These cells were then transplanted to the retina of animal models with retinal degenerations to study their therapeutic efficacy. However, because the hESCs have ethical issues and potential immune rejection, iPS cells are more promising for clinical use to repair the retinal degeneration. Using iPSC-derived RPE cells, several clinical trials are ongoing for tackling macular degeneration and other related retinopathies in the clinics (Reardon and Cyranoski 2014).

2 Derivation of Retinal Pigmented Epithelium Cells from hESCs and iPSCs

Degeneration of RPE cells mainly affects retina metabolism and normal function of photoreceptor cells and eventually causes vision loss. It was reported that dysfunction of RPE cells increases the degeneration of photoreceptor cells and retinal lesion (Tolmachova et al. 2010; Wu et al. 2016). Till date, there are no effective therapeutic approaches to stop the degeneration of RPE cells in the aging retina. As RPE plays important roles in retinal repair, different research groups have developed protocols to induce hESCs or iPSCs to differentiate to RPE cells. The embryoid body (EB) method is commonly used to generate neural stem cells and RPE cells from hESCs and iPSCs (Buchholz et al. 2009; Hirami et al. 2009; Osakada et al. 2008). hESCs or iPSCs-derived free-floating EBs are then plated onto extracellular matrix (ECM)-coated plates to promote differentiation of neural precursor cells (Carr et al. 2013). Usually this process can take 2–3 weeks to get the immature RPE and another 6–8 weeks to get the mature RPE by addition of growth factors of Activin A, TGFB1, and FGF2 to the RPE induction medium. Some studies combined EB-mediated differentiation protocol with specific growth factors and signal molecules of nicotinamide, Dkk-1, and Lefty-A to induce production and maturation of RPE (Meyer et al. 2009; Zhu et al. 2013). It was reported that activating Wnt-signaling pathway improved the RPE conversion rate to more than 90% on day 14 (Buchholz et al. 2013; Leach et al. 2015). Another approach to generate RPE is to use reprogramming to directly convert somatic fibroblasts to RPE by expressing retina-lineage-determining transcription factors. The reprogrammed cells were shown to have morphological and functional characteristics of early stage RPE cells. This method omits a pluripotent state of hESCs or iPSCs to directly generate safe and stable RPE from somatic cells to reduce the tumorigenicity of undifferentiated cells retained in the hESC- or iPSC-derived RE cells (Li et al. 2016a; Zhang et al. 2014). Recently a novel method was developed to apply a synthetic peptide-containing copolymer (Synthemax II-SC) as a support for differentiating the hESCs to RPE. This Synthemax II-SC was shown to keep stable growth of undifferentiated hESC lines and efficient differentiation of hESCs into functional RPE. These hESC-RPE cells are able to form functional phagocytosis of rod segments and secrete pigment epithelium-derived growth factors. As this method is free of animal-derived components, it is more applicable for transplanting the hESC/iPSC-RPE cells to clinical patients with retinal degenerative disorders (Pennington and Clegg 2016). To further improve the enrichment of RPE, the extracellular matrix-derived Matrigel and neutralizing factors were combined to provide a 3D environment to increase the formation of differentiated RPE from iPSC/ESCs (Zhong et al. 2014; Zhu et al. 2013). In this way, iPSC/ESC-derived RPE cells were transplanted to animal models with retinal degenerations and were found to improve the degeneration of retinal cells by protecting photoreceptors in the retina. Nevertheless, using animal products as feeder cells to culture hESCs/hiPSCs may induce some side effects, including immune-response in clinical applications (Vaajasaari et al. 2011). Thus a defined xeno-free culture system is needed in clinical application despite of the longer period and higher cost to culture the cells. To improve the growth and maintenance of hESCs/iPSCs, MEF cells can be

replaced by human foreskin fibroblasts (hFF) while KnockOut™ Serum Replacement (KO-SR) can substitute fetal bovine serum (FBS). Several studies have successfully generated functioning RPE without using FBS, murine feeder cells, and Matrigel (Plaza Reyes et al. 2016; Wang et al. 2015). The matrix proteins for RPE differentiation and enrichment can also be replaced by collagen, laminin, fibronectin, and vitronectin or mature xeno-free commercial products (Bergstrom et al. 2011; Hazim et al. 2017). Other studies explored the autologous or heterologous stem cells such as the bone marrow–mesenchymal stem cells (BM-MSC) or umbilical cord–derived mesenchymal stem cells (UC-MSC) for their differentiation to the RPE cells for the treatment of retinal degenerations (Tian et al. 2018; Wang et al. 2017). Recently an efficient method for deriving homogeneous RPE cells from pluripotent stem cells was developed in a period of 45 days on an adherent, monolayer system and defined xeno-free media and matrices. This method utilizes sequential inhibition and activation of the activin and bone morphogenetic protein signaling pathways, and can be applied to derive RPE cells from hESC/iPSCs (Choudhary et al. 2017).

No matter which approaches are used, the morphological and functional features of the differentiated RPE cells should be characterized, which include pigmentation, polarity, and the expression of specific genes at the transcriptional and translational levels. Importantly the differentiated pigmented cells should be seen as monolayer cells with cobblestone morphology appearing in the cell body. By immunocytochemistry and real-time RT-PCR analysis, these differentiated pigmented cells should express the RPE-specific genes such as *RPE65* and *CRALBP*, *MERTK* and *BEST1*, with the decreased expression of the pluripotent *OCT4* gene (Idelson et al. 2009; Kamao et al. 2014; Singh et al. 2013). Further functional analysis of differentiated RPE is needed to measure phagocytosis by purified ROS or foreign bodies, neural cell potentials by electrophysiological patch-clumps, and vision observation of animal with transplanted hESC/iPSC-derived RPE (Hazim et al. 2017; Liao et al. 2010).

3 Derivation of Photoreceptor Cells from hESC and iPSCs

It was possible to isolate the retinal stem cells/progenitors from fetal embryonic retina, which are then induced to specific retinal cells in vitro and in vivo (Klassen et al. 2004; MacLaren et al. 2006), but these cells have limited self-renewal potential. hESCs can be differentiated to any kind of retinal cells by combination of cytokines and growth factors, including BMP antagonist and Wnt inhibitor, or ectopic overexpression of retinal lineage transcription factors (Sugie et al. 2005). After intraocular injection, the in vitro differentiated hESC-derived retinal cells were able to integrate into the appropriate retina layers, and express specific markers for both rod and cone photoreceptor cells. Once the hESC-derived retinal precursor cells were transplanted into the subretinal space of a mouse model of Leber's congenital amaurosis, these cells differentiated into functional photoreceptors to improv the light response of the transplanted animals. This result demonstrated that hESC-derived retinal cells can be used for photoreceptor replacement therapies (Lamba et al. 2009). The retinal photoreceptors can also be induced from hESC-derived embryoid bodies (EB) in the culturing medium containing IGF-1, noggin, and DKK1 (Mellough et al. 2012). To form the 3D retina, hESC-derived retinal cells were induced to self-organized multilayered cell sheets, containing both rods and cones of photorecepors. These 3D structural cell sheets can spontaneously curve to produce large-scale retinal tissues for clinical use (Nakano et al. 2012).

With the fast advance in iPSC technology, different methods have been developed to induce iPSCs into RPE cells with morphology, pigmentation, formation of tight junctions, photoreceptors, and retinal ganglion cells (RGC). A clinical perspective is to induce patient-derived iPSC to differentiate to functional retinal tissues in vitro for transplantation. These retinal tissues should contain both RPE and photoreceptors with light sensitivity (Garg et al. 2017). One study reported that iPSCs were induced to multipotential retinal progenitors,

which have a range of retinal potential to generate retinal ganglion cells (RGCs) and cone and rod photoreceptors in response to stage-specific developmental signals (Parameswaran et al. 2010). Another study even demonstrated that human iPSC-derived retinal cells can recapitulate the key develepmental process of retina observed in vivo and form 3D retinal cups that contain most of all retinal cells arranged in their proper layers. Importantly, the human iPSC-derived photoreceptors in retinal tissue achieved the functional photosensitivity. This study made a good progress for the iPSC-derived retina to be used for clinical therapies (Zhong et al. 2014). The loss of RGCs is implicated in many retinal degenerative diseases. But the major difficulty is to differentiate hESCs/iPSCs to the fully functional RGCs, as the RGC differentiation is regulated by many intrinsic and extrinsic factors. One study reported a novel chemical protocol for the differentiation of hESCs/iPSCs into RGCs with an efficiency of 30%. These hESCs/iPSCs-derived RGCs were also shown to have both spontaneous and evoked excitatory postsynaptic currents, indicating their functional maturation (Riazifar et al. 2014). Recently the 3D retinal organoids were derived from hESCs/iPSCs, which can recapitulate the structural and functional retina and be used as in vitro models of retinal tissue. In addition to study the RGC development, retinal organoids can also be used to observe extensive axonal outgrowth and neural circuits in the organoids as well as to replace the degenerating neural cells of retina (Fligor et al. 2018; Zou et al. 2019).

4 Animal Studies Using Retinal Cells Derived from hESCs and hiPSCs

Since RPE cells are mainly playing supporting role for the photoreceptors of the retina, a lot of studies have transplanted RPE cell to animal models to study their protective effects on photoreceptors for improvement of visual function. Most hESC/iPSC-derived RPE can be characterized by their gene expression and functional analysis. Optical coherence tomography (OCT) and fundus photography are commonly used to visualize and track transplanted cells in the retina. After the derivation of the RPE from pluripotent stem cells, the phenotypes and functions of the differentiated RPE need to be verified in animal models of the mice, rats, or nonhuman primates before differentiated cells are transplanted to the patients. The derivation process for hESCs/iPScs to be differentiated to retinal projenitors and RPE cell, RGC, CONe, Rod cells is represented in Fig. 9.1.

The early generation of RPE from hESC was reported by Klimanskaya et al. in 2004. They used systematic differentiation protocol to derive RPE cells from hESCs, and the differentiated cells expressed specific molecular markers of RPE and made functional phagocytosis (Klimanskaya et al. 2004). Another study showed that transplantation of mouse ESC-derived neural precursor cells could increase the survival and function of host endogenous neurons including photoreceptors (Meyer et al. 2006). After that, the same group derived hESC-RPE, and the transplanted hESC-RPE cells were capable of rescuing visions in retinal spontaneous AMD rats (Lu et al. 2009; Lund et al. 2006). The long-term functional vision rescue was observed in rat model of retinal degeneration and mouse model of Stargardt disease transplanted with hESC-derived RPE. The transplanted hESC-RPE cells were capable of surviving in subretinal space of RCS rats for more than 220 days. The cells were found to sustain visual function and photoreceptor integrity was seen in retinal circuitry. Importantly no evidence of teratoma/tumor formation was observed after subretinal transplantation of hESC-RPE cells, supporting the clinical use of these transplanted cells (Lu et al. 2009). To study the structural and functional maturation of hESC-derived retinal cells in primate models of retinal degeneration, the hESC-derived retinal tissues (hESC retina) were grafted in monkeys and were observed to differentiate into retinal cells of rod and cone photoreceptors and have structured outer nuclear layers after transplantation. Further analyses suggested the formation of host–graft synaptic connections in the transplanted retina. This study demonstrated the

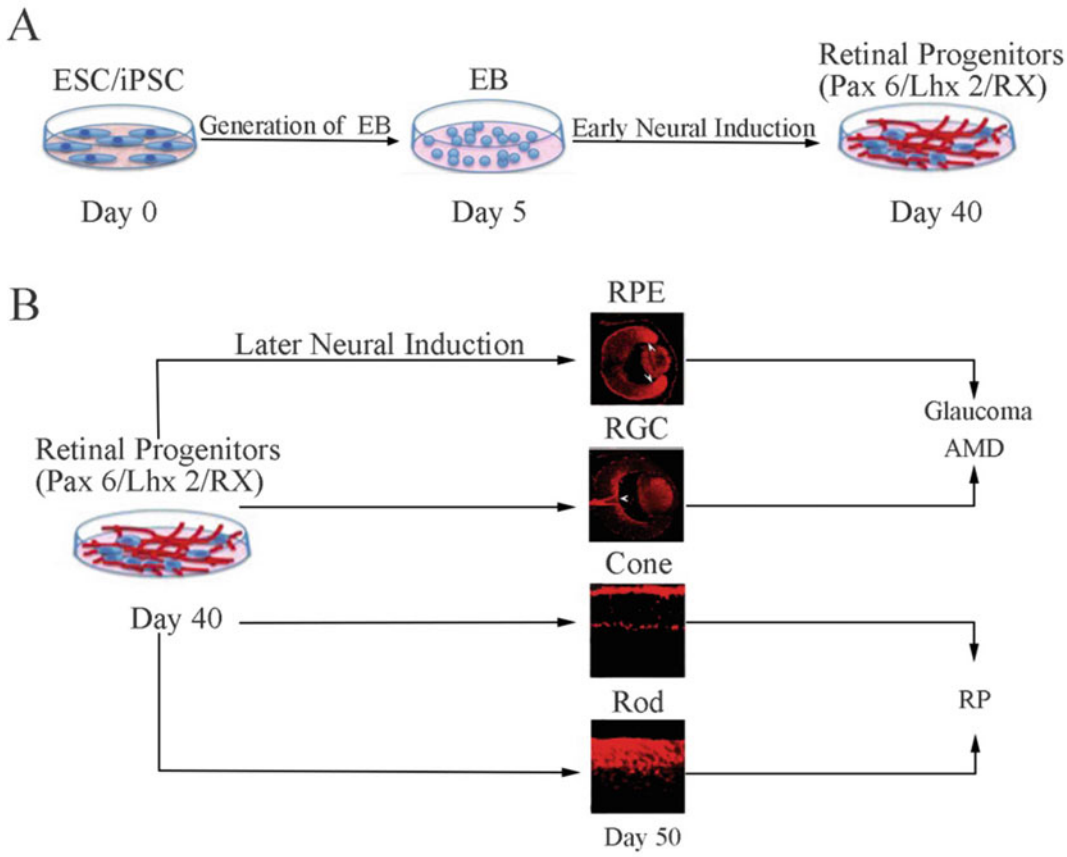

Fig. 9.1 hESCs/iPSCs differentiated to the retinal neural cells in vitro. (**a**) hESCs/iPSCs differentiated to the embryonic bodies (EB) and retinal progenitors. (**b**) Retinal progenitors differentiated to the RPE, RGC, cone and rod photoreceptors for transplantation therapy

clinical feasibility of hESC-retina transplantation for clinical applications (Shirai et al. 2016). Most studies transplanted hESC-RPE to subretinal space of retina for the improvement of vision. Other groups optimized delivery methods for transplanting hESC-RPE cell sheets to retina of animal models. To hold a monolayer of hESC-RPE cells, parylene substrate was used to support survival of hESC-RPE and was transplanted with cells into subretinal space of RCS rats to evaluate vision improvement of the transplanted rats. It was found that in addition to protecting the photoreceptor, the function of rod cells was also significantly recovered (Thomas et al. 2016). Some studies reported the generation of retinal progenitor cells (RPCs) from rat ESCs (rESCs). These rESC-derived RPCs (rESC-RPCs) were able to survive in the host retinas of RCS rats and protected the retinal structure and function of rats following the cell transplantation, as shown in Fig. 9.2 (Qu et al. 2015; Brant Fernandes et al. 2016). In porcine experiments aiming to test surgical feasibility on eyes comparable to human, transplanted ESC-RPE cells improved the vision function of the pigs. These studies not only provided safety evidence, but also provided feasible protocol for clinical trials, including required cell amount to be transplanted, as well as the cell delivery approaches, etc. (Koss et al. 2016). With the progress in somatic cell reprogramming technique to generate iPS cells, many labs have derived iPSC cells from different somatic cells and original four-gene iPSCs have been decreased to two-gene or one-gene iPSCs, which

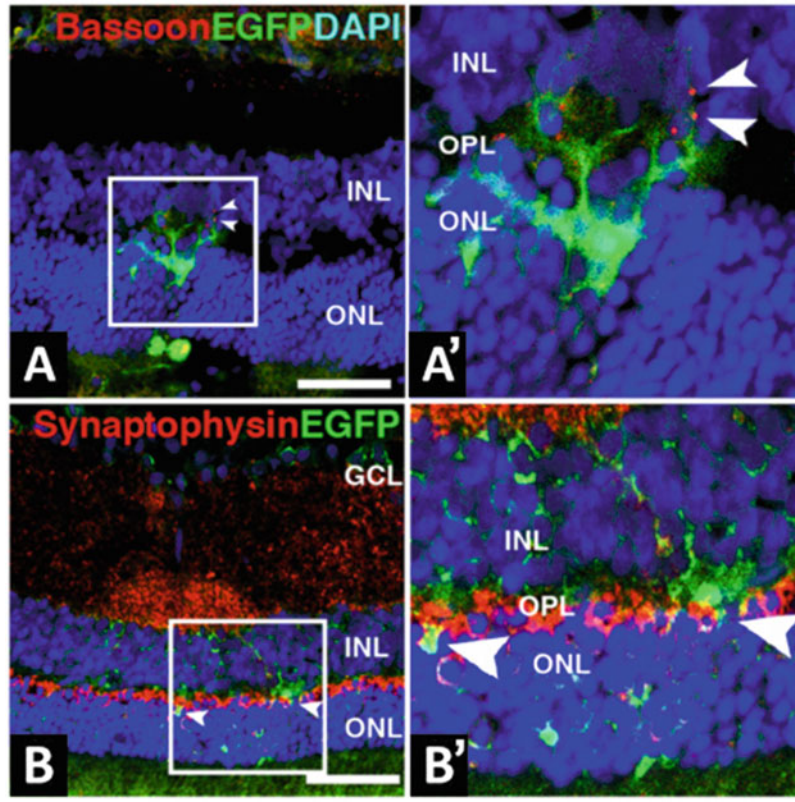

Fig. 9.2 rESC-derived RPE integrated into host retina after transplantation. (**a**, **b**) Integration of EGFP-rESC-RPE in host retinal layers. Colocalization of presynaptic markers Bassoon (**a**) and Synaptophysin (**b**) with EGFP expressed by grafted RPE cells in OPL, ONL, and INL. (**a**', **b**') The magnified images of the rectangles in (**a**, **b**). Arrow heads indicated the colocalization of EGFP-labeled grafted cells and Bassoon or Synaptophysin. *GCL:* ganglion cell layer, *INL*: inner nuclear layer, *OPL:* outer plexiform layer, *ONL:* outer nuclear layer (modified from Qu et al. 2015)

reduced the risk of tumorigenicity (Buchholz et al. 2009; Carr et al. 2009; Krohne et al. 2012). iPS cells were ever reported to spontaneously differentiate into RPE cells to form highly differentiated RPE monolayers. This study showed that iPSCs-derived RPE cells have similar gene expression and functional phagocytosis to fetal RPE and hESC-RPE cells, supporting that iPSCs are similar to hESCs in their differentiation potential(Buchholz et al. 2009). In addition, the iPSC-RPE cells from a retinitis pigmentosa (RP) patient were shown to restore visual function in a retinal degeneration mouse model with *Mfrprd6/Mfrprd6* mutations. The transplanted iPSC-RPE cells were also observed to have long-term survival in the mouse model with no tumor formation, indicating the safety and stability of these iPSC-RPE cells (Li et al. 2012, 2014). Since cellular polarity is essential for many cellular functions such as ion channels, receptors, and transporters distributed on apical side of RPE cells, Kamao et al. (Kamao et al. 2014) generated polarized monolayered iPSC-RPE sheets to increase their function for vision improvement. A study ever performed the structural, molecular, and electrophysiological comparison of 15 iPSC-RPE monolayer lines that were derived from distinct tissues of donors with different genetic backgrounds and found that function of iPSC-RPE was more significantly affected by the genetic differences of donors rather than the different somatic tissue sources (Miyagishima et al. 2016). The iPS-RPE cell sheets should meet the criteria of RPE characterization without immune-rejection or tumor formation upon autologous transplantation to a primate model. To analyze the functions of iPSC-derived RPE, a study compared the vision protective of iPSC-derived RPE with the fetal NSCs in mice with photoreceptor degeneration. It was found that human iPS-RPE cells significantly attenuated photoreceptor degeneration in 2–3 weeks after

injection into the subretinal space and survived up to at least 12 weeks. The iPS-RPE cells were shown to have better protective effects to retinal degeneration than the fetal NSCs (Sun et al. 2015). To overcome the integration of transgenes in the iPS cells, the iPS cells were produced from the patient's skin fibroblasts using nonintegrating episomal vectors, and then differentiated into RPE sheets for transplantation therapy in animal models or clinical patients (Kamao et al. 2014). Some patient-derived iPS cells may have the genetic mutations which affect the functions of iPSCs-derived RPE cells, and the mutations can be corrected by gene-editing technologies such as the CRISPRE-cas9-mediated gene editing as described in other chapters.

To convince the safety and therapeutic effects of hESC-derived retinal cells in non-primate monkeys, the hESC-derived retinal cells were labeled with green fluorescent protein (GFP) before transplantation. The 100 ul (10,000 retinal cells/uL) hESC-retinal cells were injected into the subretinal space of the monkey eyes. The differentiation and survival of hESC-retinal cells in the retina of monkey was examined 3 months after transplantation. It was found that GFP-labeled axonal projections had emanated from the transplanted cells with some projection into the host optic nerve. This study demonstrated that hESC-retinal cells injected into the submacular space of the retina survive at least 3 months posttransplantation in host monkeys without any side effects, supporting the safety of transplanting human-derived retinal cells to repair the retina of non-human primates (Chao et al. 2017).

5 Clinical Studies Using Retinal Cells Derived from hESCs and hiPSCs

Even though transplanted hESC/iPSC-RPEs showed solid efficacy and the improvement of visual functions in numerous animal models, few clinical studies have been carried out yet. One group showed the preliminary safety data of hESC-RPEs transplanted to subretinal tissue of a patient with dry AMD and another patient with SMD. The transplanted hESC-RPEs were controlled to contain more than 90% RPE cells. The visual ability was slightly improved from 0 to 5 letters in the transplanted eye of the patient with SMD, and vision also seemed to be improved from 21 to 28 ETDRS letters in the patient with dry AMD. There is no observed abnormal growth of hESC-RPE or immune-mediated transplant rejection in either patient 4 months after transplantation (Schwartz et al. 2012).

Another clinical study reported the hESC-RPEs transplantation for nine dry AMD patients and nine SMD patients. Of these 18 patients, 13 patients had been shown to have increasing subretinal pigmentation from transplanted RPE cells. Both general and peripheral vision activity was improved at different levels for most of these patients. Furthermore, near and distance vision was also improved by 16–25 points in patients with AMD and 8–20 points in patients with SMD 12 months after transplantation of hESC-RPE cells (Schwartz et al. 2015). A Korean group reported the safety and efficacy of hESC-RPE cells transplanted into subretinals of two Korean patients with dry AMD and two other Korean patients with SMD. These patients were followed for 1 year and there was no evidence of adverse proliferation, tumorigenicity, or other serious safety issues related to the transplanted cells 1 year after hESC-RPE transplantation. Visual acuity was also improved 9–19 points in three patients. These results confirmed that hESC-derived retinal cells could serve as a potentially cell source for transplantation therapy on patients with retinal degeneration (Song et al. 2015). A UK group showed the efficacy of hESC-RPE transplantation in 12 patients with advanced Stargardt disease (STGD) by subretinal transplantation of up to 200,000 hESC-RPE cells with systemic immunosuppression for 13 weeks. Even though only four patients showed improved visual acuity 12 months after transplantation of hESC-RPE, no hyperproliferation and inflammatory responses were found in any of 12 patients, supporting the safety of subretinal transplantation of hESC-RPE cells (Mehat et al. 2018). The autologous iPSC-RPE was first transplanted to an elderly female patient with advanced AMD

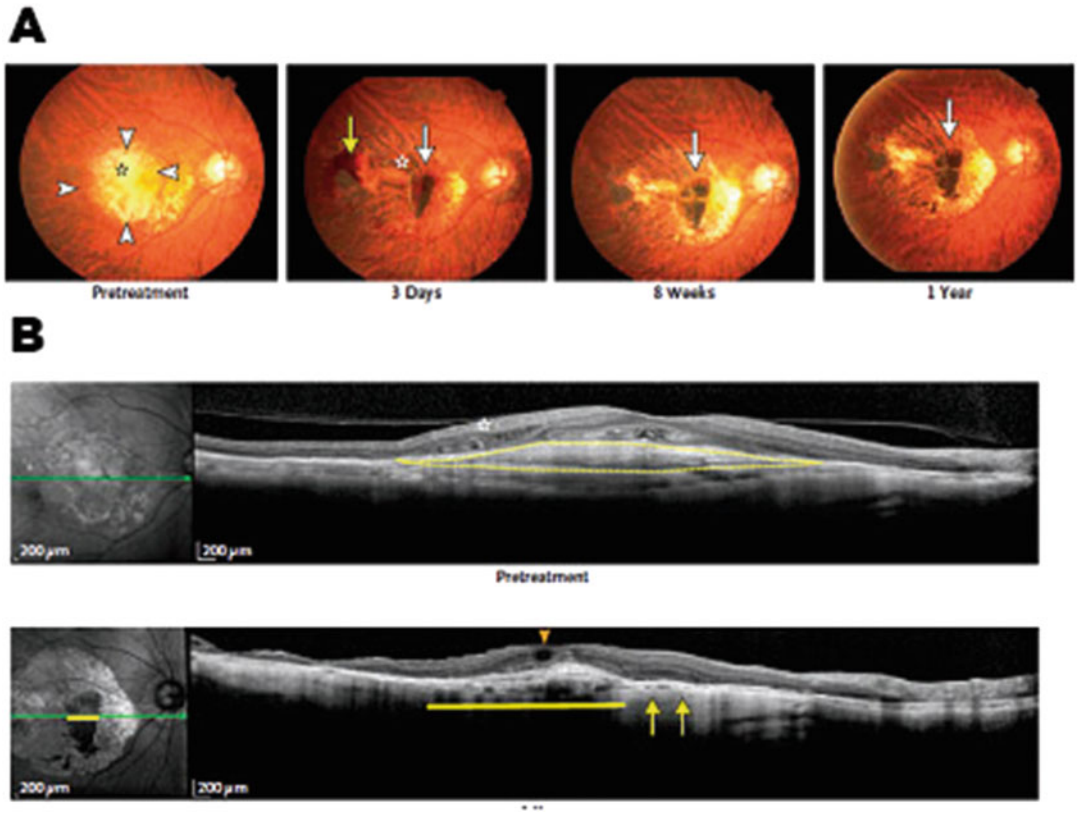

Fig. 9.3 The autologous iPSC-RPE sheet was transplanted to the retina in a patient with AMD. Panel (**a**) The left-most image shows a large fibrotic neovascular membrane (black asterisk, white arrowheads indicating the margin). After the removal of neovascular membrane, the underlying choroid vessels became readily visible and were almost intact (white asterisk in the left-middle image). Hemorrhages were observed at the graft insertion site 3 days after surgery but were absorbed in 2 weeks (yellow arrow, the second image from left). Two middle images and the right image showed the graft sheet was curled and then flattened by 8 weeks (white arrows). Panel (**b**) shows vertical sections by optical coherence tomography (OCT) before and 1 year after surgery. Upper image: the neovascular membrane was observed as a dense hyperreflective mass under the macula before surgery (marked by the yellow dotted line). The tubules of the photoreceptor cell layer formed as rosette-like structures in the fovea (asterisk, upper image). At 1 year, a highly reflective RPE-like cell line extended nasally from the graft sheet and the structured photoreceptor cell layer and choroid space were retained above and below the line, respectively (yellow arrows, lower image). This figure is modified from Mandai et al with permission of reprinting. (2017)

in 2014. A recent clinical study transplanted the autologous iPSC-derived RPE cell sheet into the right eye retina of a patient with advanced neovascular AMD. After 3 months, the patient's vision was found to be improved. One year after transplantation, no serious complications and rejections were observed, suggesting that autologous iPSC-RPE cell transplantation was safe and had effective treatment on AMD, as shown in Fig. 9.3 (Mandai et al. 2017). Recently a study transplanted a synthetic parylene substrate to support the monolayer hESC-RPE cells in subretinal layers of patients with advanced AMD to assess the safety and efficacy of hESC-RPE cells. As a result, some patients had improved visual function by 17 points, and none of the implanted eyes showed progression of vision loss (Kashani et al. 2018). There are two ongoing iPSC-RPE-based clinical trials (NCT02162953, NCT02464956) to investigate bestrophinopathy and dry AMD respectively, which may provide more evidence of safety and

efficacy of iPSC-RPE in the future. Currently transplantation of hESC/iPSC-derived RPE in phase I and phase II clinical trials for retinal degeneration is using the synthetic substrates to support monolayer cell sheets of RPE for transplantation. A major problem is that these substrates may cause local inflammation and fibrosis in animal models or clinical trials due to longer degradation times. Thus, more thinner fibrin hydrogels were developed as a support material for the RPE transplantation and showed that these fibrin hydrogels support growth of stem cell–derived RPE and are easily degraded within hours without damage to the RPE sheet. These fibrin hydrogels provide clinically suitable support for transplant RPE to patients with retinal degeneration diseases (Gandhi et al. 2018).

6 Future Directions

Using hESC/iPSC-derived retinal cells to repair degenerated retina seems promising, as the grafted retinal cells have the ability to form outer photoreceptor cells and inner RPEs against host inner retina. Numerous studies have achieved progress to reach this goal. Some studies reported generation of retinal cup to contain all major retinal cell types in proper 3D configuration and were able to produce photosensitivity after being grafted to rat models with advanced retinal degeneration (Assawachananont et al. 2014; Nakano et al. 2012; Zhong et al. 2014). However there are some complications in hESC/iPSC-related transplantation. These include gliosis of transplanted cells into the retina, epigenetic modification, and genome instability of iPSC-derived retinal cell lines. The latter may be ameliorated by utilizing a combination of transgene-free methods to generate the safe iPSC lines (Watanabe et al. 2013; Ye et al. 2016). There are some major obstacles to be overcome before iPSC-derived retinal cells are translated to the clinic. One concern is tumorigenesis of iPSC-retinal cells due to the reprogramming methods and residues of undifferentiated iPSCs. The chances of tumorigenesis have been greatly reduced with novel reprogramming methods, which include episomal plasmids and small molecule–mediated reprogramming that are not integrated into the genome (Ito et al. 2012; Yang et al. 2017). The remaining undifferentiated cells can be overcome by optimizing the differentiation protocol and sorting the differentiated retinal cells through flow cytometry or other sorting techniques (Collin et al. 2016; Zou et al. 2019).

Another challenge facing stem cell transplantation is the potential of immunogenicity of allogenic hESC- and iPSC-derived cells. Future studies should investigate into different cocktails of culture conditions and their effects to cell genetic and epigenetic integrity with whole genome sequencing, whole-genome methylation and transcriptome expression analyses, as well as to look into the molecular basis behind these aberrations. The robust short-term safety data provided by hESC-based phase I or II trials encourages further investigation into the hESC- and iPSC-derived-RPE replacement therapy. Follow-up of the ongoing trials will provide more valuable evidence for long-term safety and improvement of vision acuity of transplanted hESC- and iPSC-derived retinal cells in patients with retinal degeneration disorders.

Acknowledgments This work was supported by National Natural Science Foundation of China (NSFC 81571241). We also thank Wei Wang, and Yanming Liu in our lab for technically editing the references and preparing figures of the manuscript.

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Future Challenges and Perspectives for Stem Cell Therapy of Neurodegenerative Diseases

10

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1 Introduction

As we discussed above, stem cell transplantations have made great progress for treating patients with neurodegenerative diseases (NDs). With the fast growth of stem cell research, various stem cells have been studied and can be induced to specific types of neurons and glial cells with high purity in vitro. A large number of preclinical studies confirmed that transplanted embryonic stem cells (ESCs) and induced pluripotent stem cell (iPSC)–derived neural cells, including neural stem cells (NSCs), neural progenitor cells (NPCs), neurons, and glia cells, can survive and maturate to functional neural cells in animal models of Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), multiple sclerosis (MS), retinal degeneration (RD), and other diseases. However, clinical applicable stem cell–derived neurons such as specified NSCs, dopaminergic neurons, motor neurons, and interneurons for transplantation to the patients need to be further investigated, including substantial evidence that the transplanted cells can form connections with neurons in the host brains, restore release of neurotransmitters in vivo, and significantly improve neurological deficits that are similar to the symptoms of patients with different NDs.

2 Specific Neural Cell Types Are Needed for Different NDs

Neural stem cell replacement therapy would enable the transplanted cells to be distributed through the affected neural tissues from the injection sites while their functional integration into the host neural circuitry can be established and their resistance to the pathological environment that causes the neural cell degeneration can be maintained. In certain neurodegenerative diseases, especially AD or MS, multiple pathogenic factors are involved, which affects multiple neural systems simultaneously (Choi et al. 2014; Scolding et al. 2017). Thus, the required neural stem cells must be cultured and expanded in vitro to obtain sufficient numbers to achieve a good level of engraftment that makes those cells suitable for the clinical use (Huang et al. 2020).

In addition to the functional release of the required neural transmitters, the transplanted

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F. Han, P. Lu (eds.), *Stem Cell-based Therapy for Neurodegenerative Diseases*, Advances in Experimental Medicine and Biology 1266, https://doi.org/10.1007/978-981-15-4370-8_10

cells have to be integrated into the host central nerve system. Some transplanted neural stem cells or neurons could also be genetically engineered to secrete/produce the neurotrophins that can support the long survival and defined differentiation to the required cells after transplantation. These cells should retain their ability not only to modulate the host neural network, but more importantly to integrate into the host neural circuits. For some neurodegenerative diseases, the NSCs derived from fetal brains or reprogrammed somatic cells could be engineered to secrete dopamine, choline acetyltransferase (ChAT), gamma aminobutyric acid (GABA), or inhibitory signaling molecules to maintain the inhibitory function of the brain networks. In the case of ESC- and iPSC-derived cells, every cell type needs to be identified in the culture, especially the number of neural cells, non-neural cells, and undifferentiated precursor/stem cells. For some diseases, such as AD and ALS, the appropriate donor cells to be transplanted are still unclear and one or more specific neurons or glial cells for transplantation will need to be identified in further studies (Duncan and Valenzuela 2017; Mazzini et al. 2018; Jiang et al. 2020).

The pluripotency of hESCs and iPSCs indicated that these cells can be differentiated to any kind of cells for cell replacement therapies in NDs (Hallett et al. 2015). Because of variations in reprogramming protocols, differentiation methods, and individual difference, iPSCs vary in term of their differentiation efficiencies and genetic backgrounds, which definitely affect their clinical applications for transplantation therapy (Berry et al. 2018).

Recently, studies showed that fibroblasts can be directly reprogrammed to generate induced NSCs (iNSCs) or induced neurons (iNs) (Han et al. 2012; Lujan et al. 2012; Velasco et al. 2014). iNSCs or iNs can also be generated from human astrocytes and other cell types. These studies mainly used miRNAs or the lentiviral or retroviral plasmids to express the transcription factors which control the development of specific neural lineages to induce the transdifferentiation of somatic cells (Abernathy et al. 2017; Lim et al. 2015). Some studies reported that transplanted iNSCs can survive in vivo at least 6 months (Maucksch et al. 2013; Zhou and Tripathi 2012). However, only some of these studies demonstrated authentic differentiation of neural cells in vivo of the iNSCs or iNs after transplantation. Some studies did not show any improvement of behaviors after transplantation to the animal models. In additon to use the transplanted cells for repairing the degenerated neurons in NDs, recent studies have shown the muse cells in the central nerve system can be activated for the treatment of NDs (Leng et al. 2019). Because these approaches can generate autologous and patient-specific iNSCs or iNs for potential neural cell replacement therapies in NDs and other related disorders, further studies are necessary. Most recently for the first time the induced neural precursors (iNPs) were derived from healthy adult dermal cells through non-viral expression of lineage factors, which can give rise to dopamine neuronal-like cells, suggesting reprogramming could be a suitable strategy for modeling and treating neurodegenerative diseases using aged donor-derived cells (Maucksch et al. 2013; Playne et al. 2018). Thus, high efficient methodology is needed for consistent generation of a desired neural cell type for the treatment of different NDs.

3 Progenitors with Proliferation Capability or Postmitotic Neural Cells for Transplantation

With cell transplantation it is expected that the grafted cells are able to have functional properties of the original host cells to induce substantial benefit for treating NDs. This requires that the transplanted cells should be differentiated to mature neurons. As the mature neural cells are easy to die in the new host microenvironment after transplantation, most studies transplanted the fate-determined neural stem cells such as the dopaminergic progenitor cells (DAPCs) for treatment of PD expecting the subsequent maturation of DAPCs to dopamine neurons. For some diseases, it may be ideal for transplanting immature cells, such as neural stem cells, that divide a

few times before becoming neural cells that can be integrated into the host neural circuit. The advantage of the immature cells is that the transplanted neural stem cells/progenitor cells have capacity to increase survival and differentiate to the specific neurons. But uncontrolled differentiation of transplanted stem cells could introduce variability among patients and may be even harmful for patients in some cases. Thus the iPSC/ESC-derived neural stem cells/progenitor cells need to be sorted or purified before transplantation.

Each kind of neurodegenerative diseases has a major type of neuron affected. For PD, the dopamine neurons are the causative cells, whereas in ALS the motor neurons are mainly degenerated. For AD, even though the hippocampus and cerebral cortical layer are the major affected brain regions, several other parts of the brain are also found to be involved. The cholinergic neurons may be the main neurons affected, but other types of neurons such as the 5-TH+ neurons and glial cells are also affected. Thus the neural stem cells and different neuronal types should be included in transplanted cells to get optimized therapeutic efficacy. As the main affected neurons are GABA+ interneurons for HD, the hESC/iPSC-derived cells or iNPCs should differentiate into striatal GABA+ interneurons in vitro and then be transplanted to patients to achieve optimal function, the nonstriatal phenotype cells should be eliminated.

4 Cell Compatibility of Patients and Immune Rejection of Stem Cell–Based Therapies

In order to achieve therapeutic efficacy of cell transplantation, donor cells need to be human leukocyte antigen haplotype–matched with the recipient, and nonmatched recipients require immunosuppression in order to prevent rejection of the transplanted cells. Ideally, it will be useful for some iPS cell banks or the bone marrow or umbilical cord banks to have isogenic and patient-specific or human leukocyte antigen–matched iPSC- or iNSC-derived cells for transplantation without immunosuppression. In the studies of transplanting fetal midbrain dopaminergic cells for PD patients, the grafted cells were reported to survive in brains of patients for up to 14 years with only a 6-month-long immunosuppression that was sufficient for such a prolonged survival. Nevertheless, new approaches are still needed for future stem cell–based therapies in NDs to enhance compatibility of donor cells with patients and suppress immune rejection of transplanted cells. In particular, the iPSC-based cell therapy needs to be performed individually.

5 Regulatory Standards of the Transplantable Cells for Clinical Use

Eventually, good manufacturing practices (GMP) guideline should be set up to ensure that the transplantable cells for clinical use are consistently produced and controlled. Because NDs usually are relatively slow-progressive diseases, it will take many years for clinical trials to demonstrate that cell therapies can success to halt or reverse disease progression. The safety and ethical concerns of stem cell therapies, especially for NDs, will likely be addressed and tightly controlled. However, since stem cell-based therapy is unique, new regulatory policies need to be developed to foster its appropriate development and successful usage for clinical application.

6 Future Directions

In the past two decades a lot of studies indicated that human stem cell–derived neural stem cells or neurons can survive and improve the functional defects in animal models of NDs. However, more preclinical research work has to be done in further studies before these cells are translated to the clinics. One of the major concerns is that if they can substantially integrate into the host brain to form neural circuit in vivo to dramatically improve neurological deficits that are similar to the symptoms of patients. The connectivity between transplanted cells and the host cells needs to be clearly demonstrated by standard

immunohistochemical labeling in combination with techniques of anterograde and retrograde tracing. Secondly, the transplanted cells should be functional in appropriate small and large animal models, such as the unilateral 6-OHDA-lesioned rat model as well as the monkey model for PD, double transgenic APP/PS1 mouse model for AD, and transgenic SOD1 rat model for ALS. The effects of sustained functional improvement by grafted cells in the animal model of NDs must be demonstrated by two or more standard behavior tests. Thirdly, the transplanted cells must survive for a long term and can differentiate into the appropriate phenotypes of neurons, such as midbrain dopaminergic neurons for PD or forebrain cholinergic neurons for AD, and spiny striatal projection interneurons for HD. The pattern of cell migration should be documented besides their connectivity with the host. Although grafted NSCs or progenitors can divide in vivo for certain time after transplantation, the cells should stop cell division after a few months and are able to differentiate into postmitotic neurons and glia without continuous cell proliferation. The grafted neural cells, including neurons and glia, should be able to elicit sustained behavioral improvements, which includes the following deficits in animals with PD as an example: paw reaching with the contralateral paw; poor performance on sensorimotor integration tasks, such as contralateral neglect or a lateralized choice reaction time task; beneficial effects in locomotor activity; and alleviation of deficits in a relevant cognitive task sensitive to fronto-striatal dysfunction, such as delayed alternation (Lindvall et al. 2012). Finally it should be intensified that the iPS cell–derived neural cells or direct induced neurons (iN) will have invaluable clinical use for replacing the lost neural cells in NDs after the tumorigenesis of iPS cells is overcome by elimination of contaminated pluripotent stem cells through cell sorting or use small molecule–induced reprogramming to generate non-tumorigenesis iPS cells.

Clinical used cells should have evidence of efficacy and safety in preclinical studies, as required for all sources of stem cells and follow the established standards as we discussed in chapter 2. Some clinical trials have achieved exciting results to use the iPSC-derive retinal cells for the treatment of retinal degenerations (Araki et al. 2019; Mandai et al. 2017). As most patients with neurodegenerative diseases have few or no therapeutic treatment options, they are usually willing to test some new approaches such as the stem cell transplantation. Therefore, scientists, clinicians, regulators, and ethicists must work together to propose the responsible guidelines for translation of stem cell research into clinical applications for patients with these diseases.

Acknowledgement This work was supported by National Natural Science Foundation of China (NSFC 81571241), Department of Science and Technology of Shandong Province, China (2017GSF18104), and Basic Research Fund from Natural Science Foundation of Shandong Province, China (ZR2019ZD39), Merit Review Award Number I01 RX002264-01A2 from the United States (U.S.), and Department of Veterans Affairs Rehabilitation Research and Development (RR&D) Service and NIH (R21 NS103074-01).

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