Human Pluripotent Stem Cells and Neural Regeneration

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What You Can Learn in This Chapter

What are the cellular and molecular processes underlying neural developmental events during embryogenesis? How can we manipulate a human pluripotent stem cell to a neural lineage of interest? Can the *in vitro* generated neural cells hold the potentiality for replacement therapy in neurological disorders?

9.1 Summary

Neurological disorders always end up with neuronal loss and neural circuit dysfunction, which could not self-repair since local neural progenitors only generate restricted neuronal subtypes, and these progenitor cells also decline remarkably during aging. Human pluripotent stem cells (hPSCs) are a valuable cell source to produce almost the entire spectrum of regional neural progenitors and then different neuronal subtypes, which showed promising potentials to replenish defined neuronal loss and restore functional neural circuits in animal models (Thomson et al. 1998; Zhang et al. 2001). Stem cell-based replacement therapy for several neurological disorders is now undergoing intensive clinical observations. This chapter mainly focuses on cellular and molecular aspects of neural development, strategies of converting human pluripotent stem cells to desired neuronal subtypes, and exemplifications of applying human pluripotent stem cells to treat neurological disorders, such as Alzheimer's disease, Huntington's disease and Parkinson's disease.

9.2 Human Pluripotent Stem Cells

Human pluripotent stem cells (hPSCs) include human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), which can self-renew or differentiate into all three germ layers under proper culture conditions. In 1998, Thomson et al., successfully isolated the cells within the inner cell mass from the early human blastocyst and long-term maintained them as hESCs on mouse embryonic fibroblast feeder layer in the presence of serum replacements supplied with basic fibroblast growth factors (FGF2) (Thomson et al. 1998). Further studies identified the essential roles of transcription factors, mostly Nanog, Oct4 and Sox2, which activate the network of pluripotency genes and repress lineage differentiation genes, and therefore keep the hESCs in an undifferentiated state. Later on, through expressing of Oct4, Sox2, Klf4 and c-Myc in differentiated somatic cells, hiPSCs were established after being given the powerful reprogramming capabilities of these pluripotency factors. Genetic, epigenetic and functional assays confirmed that hiPSCs largely resemble the nature of hESCs.

As *in vivo*, a fundamental aspect of hPSCs is their potency to generate the whole spectrum of fully specified functional cell lineages of ectodermal, mesodermal or endodermal origin. Dynamically monitoring the *in vitro* differentiation process in combination with gene targeting techniques is now an alternative way to study key developmental events during cell fate conversion, which adds enormously to the traditional paradigm in studying development via animal models. *In vitro* specified human cells also provide a previously unachieved cell model for pathologic study and phenotypic drug screening for genetic disorders. Eventually, yielding functional cell types and treating patients bearing currently incurable diseases will be one of the most important applications for hPSCs.

9.3 Neural Development

In order to fully apply hPSCs for studying development and disease, it is crucial to build differentiation protocols for various lineages of all three germ layers. The ideal protocols for lineage specification should be chemically-defined, highly efficient and faithfully mirror general principles of *in vivo* development.

Mammalian neural development could be roughly discriminated into three stages, neural induction, regional patterning as well as neurogenesis or gliogenesis. Neural induction is a process happening in a gastrulating embryo, where the upper tissue layer (epiblast) becomes thickened and flattened, and develops into the neural plate comprising columnar neural epithelial cells (Sasai and De Robertis 1997). The neural plate is the anlage to form the future entire central nervous system (CNS), which later on will become the brain and the spinal cord. On the bilateral edges of the neural plate, there are neural plate borders. During neurulation, the neural plate borders lift upwards and converge at the dorsal midline to form the neural tube. Regional patterning happens concomitantly when the neural tube forms. Regional neural progenitors (NPs) will be specified from the primitive neural epithelial cells in the neural plate along both the anteroposterior (A-P) and dorsoventral (D-V) axes. In the dorsal midline region of a closed neural tube, converged neural plate borders become the roof plate. Meanwhile, following an epithelial to mesenchymal transition, neural crest cells delaminate from the roof plate, which are primary origins of the peripheral nervous system (PNS), including the cranial, spinal and autonomic nerves as well as Schwann cells and pigment cells (Fig. 9.1).

The notochord, a transient rod-like structure of mesoderm origin, locates ventrally to the midline of the posterior half of the neural tube. The notochord plays an important role in maintaining the left-right asymmetry and development of adjacent tissues. Given signals secreted from the notochord, the adjacent ventral midline neural epithelial cells of the neural plate or neural tube will be specified into the floor plate (FP). The FP of the midbrain harbors neurogenic activity and is the major region to generate dopaminergic neurons.

Converting pluripotent epiblast cells to neural epithelia, and specifying neural epithelia into various regional neural progenitors during early neural development could be summarized into an activation-transformation paradigm (Chi et al. 2017). The stem cell located at a higher hierarchy will adopt or activate a prominent cell fate which does not require additional inductive signals, while additional developmentally related signals must be in place in order to guide or transform the stem cell to other different cell fates. During neural induction, the epiblast will adopt a neural fate by default, and Wnts and

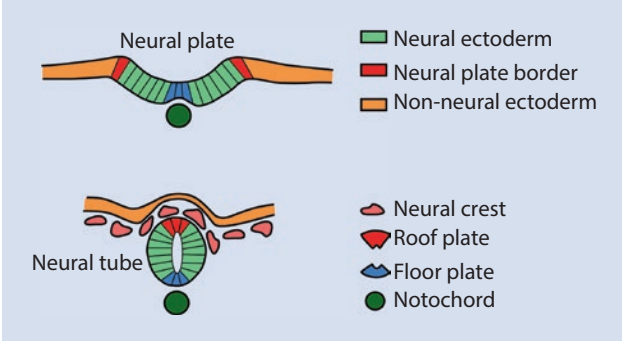

Fig. 9.1 Neural induction and regional patterning are two major cellular events accompanying neural plate and neural tube formation during embryogenesis

transforming growth factor β (TGFβ) superfamily members drive the epiblast to mesodermal, endodermal, and non-neural ectodermal tissues (Chambers et al. 2009). As to A-P patterning, neural ectodermal cells will take a prospective forebrain regional identity, while caudalization signals, including fibroblast growth factor 8 (FGF8), Wnts and retinoic acid (RA) generate midbrain, hindbrain and cervical spinal cord progenitors accordingly (Metzis et al. 2018). It has also been well demonstrated that in human cells, D-V patterning of the forebrain and spinal cord also follows the activation/transformation model (Chi et al. 2017). Both the human forebrain and spinal cord adopt dorsal telencephalon or dorsal spinal cord fate in the absence of additional inductive cues, and sonic hedgehog (Shh) is a robust and required signal morphogen to ventralize the forebrain and spinal primordium to ventral telencephalon and the ventral region of the spinal cord (Li et al. 2005; Li et al. 2009).

The activation-transformation paradigm can be partly explained by preset expression of key intrinsic transcription factors in uncommitted stem cells. Inner cell mass and epiblast cells express Sox2. During the neural induction stage, Sox2 is maintained in neural ectodermal cells within the neural plate, but shuts off when the pluripotent cells are signaled to a mesodermal or endodermal fate (Ying et al. 2003). Otx2 is a hallmark gene expressed restrictedly in the anterior neural tube, including the forebrain and midbrain, but not the hindbrain. Otx2 is also early expressed in the epiblast and neural ectodermal cells, and caudalization signals downregulate Otx2 expression and are indispensable for midbrain and hindbrain regional specification. Another striking example is related to the D-V patterning of human forebrain. Human neural ectodermal cells uniformly express Pax6, a powerful transcription factor for specification of the dorsal fate of the mammalian telencephalon (Zhang et al. 2010). Without Shh, Pax6 represses developmentally related ventral genes and therefore specifies and maintains the neuroectodermal cells to a dorsal telencephalic fate (Gaspard et al. 2008; Chi et al. 2017). The ventralization morphogen Shh represses Pax6 expression and in turn activates ventral gene expression to specify the ventral telencephalon via this repression-release model.

Within the activation-transformation paradigm, inductive signals to transform a preset cell fate to another are mostly secreted from the patterning centers, such as the roof plate, the FP, and the notochord. The notochord is a major patterning center for producing Shh. Shh emitted from notochord induces the FP in a neural tube, which also produces Shh to strengthen the ventralization magnitude (Ericson et al. 1995). In Shh null mouse, all ventral telencephalic NPs are missing as assessed by a loss of expression of all ventral markers including Nkx2.1, Dlx2 and Gsx2. Moreover, Shh represses the expression of various transcription factor genes related to the dorsal development. After neural tube closure, Wnts and bone morphogenetic proteins (BMPs), derived from the roof plate and cortical hem participate in the maintenance of the dorsal identity. Activation of Wnt signaling in the mouse ventral telencephalon also represses the subpallium development (Wilson and Rubenstein 2000). It is thought that the combined effects of the gradient concentrations of Wnts and BMPs from the roof plate as well as Shh from the FP regulate the overall D-V patterning through tight regulation of region-specific transcription factors, in particular, Pax6 and Nkx2.1. Transgenic animal studies also reveal a mutual repression between these region-specific transcription factors. Pax6 loss of function results in abnormal expression of ventral marker genes in the dorsal territory, whereas loss of Nkx2.1 results in a ventral to dorsal respecification as evidenced by extended expression of Pax6 in the ventral telencephalon. FGF8 and Wnt1 secreted from the isthmic organizer located between the midbrain and hindbrain, and the RA synthesized within the

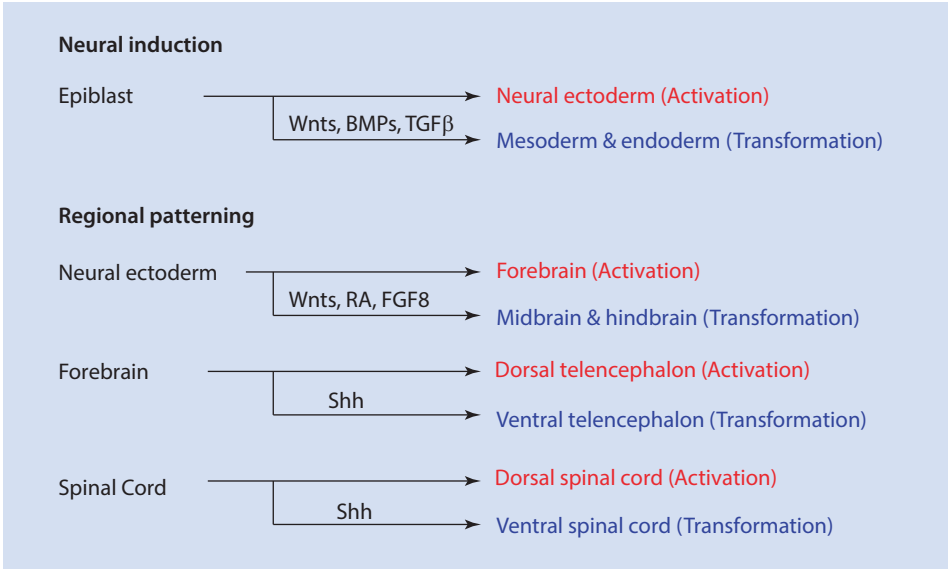

Fig. 9.2 Cell fate conversion during neural induction and regional patterning follows an activation/transformation paradigm

hindbrain are two potent caudalization signals, which are crucial for the normal patterning and development of the midbrain and hindbrain (Fig. 9.2).

Neurogenesis continues throughout embryonic development and postnatal life. Usually, a neuronal subtype is determined by the regional identity of its parental NP. NPs can self-renew for several rounds and then exit the cell cycle and differentiate into neurons and glia, including astrocytes and oligodendrocytes, sequentially. Differentiated neurons will then migrate to their destinations and integrate to form functional neural circuits, which is the basic unit to conduct a specific neurological activity. As to the cortical development, the rapidly dividing NPs locate in the ventricular zone/subventricular zone of the brain during the neural development, and subsequently differentiate into various cells in the cortical plate of the cortex. The neurogenesis in the cortex follows an "inside-out" pattern of morphogenesis: The neurons born from cortical progenitors with early birthdays tend to migrate shorter distances and those with late birthdays migrate further. During the migration, neurons are guided by the radial glial cells, which extend processes from the inner to the outer surface of the cortex. In the ventral part of the telencephalon, NPs in the medial ganglionic eminence (MGE) are the major source to produce GABAergic interneurons. GABAergic interneurons migrate tangentially in a long distance to the dorsal telencephalon, where they rearrange and mature, and form inhibitory synapses with local excitory cortical neurons.

9.4 Targeted Neural Differentiation

Both hESCs and hiPSCs are able to sequentially differentiate into neural ectoderm, regional NPs and various neurons and glia *in vitro* (Zhang et al. 2001). More importantly, the differentiation processes *in vitro* mirror exactly the developmental events happened

during embryogenesis, and the defined developmental principles are the key to guide *in vitro* differentiation. Though multiple differentiation protocols exist, hPSCs are in general first guided to neural ectodermal cells under either serum-free culture conditions or in combination with small molecules to inhibit TGFβ/BMP signaling in either suspension or adherent culture conditions (Zhang et al. 2001; Chambers et al. 2009). One important aspect of neural ectodermal cells is their responsiveness to patterning morphogens, inductive signals secreted from patterning centers. These patterning signals will therefore regionalize neural ectodermal cells to various regional NPs, which will be determined to specific subtype neurons and glia.

Both suspension embryoid body (EB) formation and dual-Smad inhibition-based adherent culture (AD) paradigms are now widely used for generation of neural ectodermal cells from hPSCs. The EB formation method suspends detached hPSCs to mimic gastrulation in the hPSC culture medium followed by the neural medium for neural lineage enrichment. While in the hPSCs-AD differentiation paradigm, inhibitors of both TGFβ and BMP signaling pathways were added to trigger neural induction. Though there is a difference between these two distinct methods, neuroectoderm cells derived from both protocols show high potency to generate different regional progenitors in response to patterning morphogens (Chi et al. 2016). Similar to the *in vivo* development, neural ectodermal cells will automatically adopt an anterior dorsal fate in the absence of additional patterning morphogens. As a potent caudalization patterning morphogen, RA efficiently caudalized neural ectodermal cells to a hindbrain and cervical spinal cord identity in both EB and AD differentiation paradigms (Li et al. 2005).

Recent research indicates that the caudal part of the spinal cord develops from neuromesodermal progenitors (NMP), which adopt a posterior regional identity even before the neural ectodermal fate has been initiated (Metzis et al. 2018). It seems that the entire epiblast could be subdivided into an anterior and a posterior compartment. It is more likely that the EB differentiation protocol favors the generation of an anterior neural ectoderm. Shh ventralizes this anterior neural ectoderm to a ventral telencephalic fate, such as the lateral ganglionic eminence (LGE) and MGE (Ma et al. 2012; Liu et al. 2013). FGF8 and Wnts regionalize the neural ectoderm generated from the EB method to a midbrain fate. However, hPSCs are easily guided to the posterior developmental structures under the AD differentiation paradigm. Under AD differentiation, early exposure of Shh causes targeted differentiation of hPSCs to a FP fate (Nkx2.1+/Sox1−/FoxA2+), while under the EB conditions, Shh activation determines a MGE fate (Nkx2.1+/Sox1+/FoxA2−) (Fasano et al. 2010; Chi et al. 2016). Spinal motor neurons are generated from the AD conditions with a much higher efficiency as compared with the EB conditions. One can expect that under the caudalization signal, hPSCs will be guided to the posterior epiblast and NMPs, which will generate more caudal spinal cord progenitors as well. Since obtaining disease related NPs and neuronal subtypes is the key for modeling or treating specific neurological disorders, EB *vs* AD differentiation paradigms need to be carefully selected.

The gradient of the patterning morphogens are crucial for establishing a regional identity of the neural progenitors. In *in vitro* neural differentiation, concentrations and durations of the patterning signals applied are equally critical. For example, medium level of Shh (200 ng/ml) treatment in neural ectodermal cells yielded from the EB method will end up with a LGE fate, while a higher level of Shh (500–1000 ng/ml) treatment in the same cells will generate more ventral MGE fate (Ma et al. 2012; Liu et al. 2013; Maroof et al. 2013). As aforementioned, NPs will sequentially generate neurons and glia. Oligo2+ ventral spinal NPs generated from hPSCs will first differentiate into the spinal motor neu-

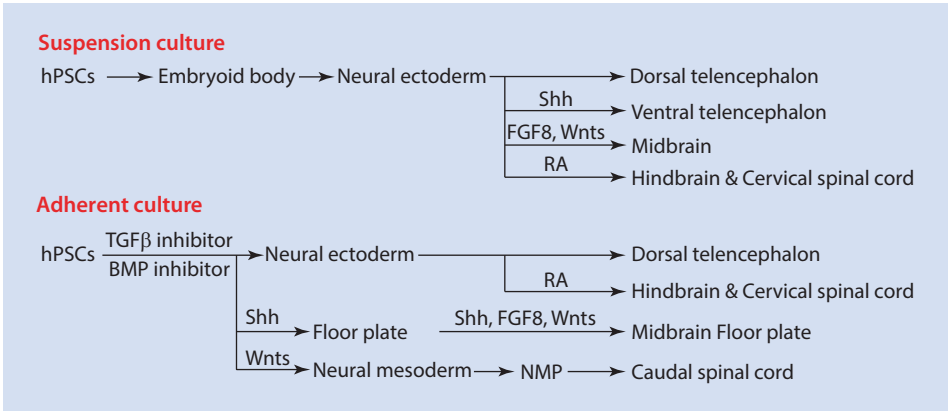

Fig. 9.3 Targeted neuronal differentiation through suspension culture or adherent culture in combination with patterning morphogens

rons in the first month of differentiation, but will generate oligodendrocytes when the progenitors are maintained for 3–6 months *in vitro* (Fig. 9.3).

9.5 LGE Progenitor Differentiation and Huntington's Disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder with distinct symptoms, including chorea and dystonia, sleep disorders, motor dysfunction, cognitive impairment, and psychiatric abnormalities. Genetically, HD is caused by the expansion of Cytosine, Adenine, and Guanine (CAG) repeats near the start of exon 1 in the gene encoding the protein Huntingtin (HTT). The mutant HTT (mHTT) proteins are detrimental for the GABAergic striatal medium spiny neurons (MSNs) in the basal ganglia, which constitute 95% of all neurons in the striatum.

Recently, cell replacement therapies represent a promising direction for the treatment of HD (Ma et al. 2012). As a proof-of-concept study, fetal neural tissue specimens isolated from donor fetal brain had been used as neuro-grafts into HD patients in clinical trials, which showed moderate improvement in motor function. Though promising, this cell replacement therapy based on fetal tissues is technically limited because of a lack of enough donor tissues, poorly defined cell types and ethics issues. Several pioneering studies have successfully specified hPSCs into LGE progenitors and functional striatal MSNs. This is mostly achieved by applying medium level of Shh in human neural ectodermal cells under EB culture conditions. Cocktailing the Activin A and Wnts signaling shows an improvement in generating LGE progenitors. The LGE progenitors yielded from hPSCs are positive for the forebrain marker FoxG1, and LGE marker Meis2 and Gsx2. MSNs generated from these LGE progenitors *in vitro* have typical spiny morphology and uniformly express DARPP32 and GABA. More importantly, MSNs differentiated from hPSCs show gradual maturation and have activity-related neurotransmitter release, spontaneous action potentials and synaptic connections *in vitro*. Grafting of hPSCs differentiated LGE progenitors in either surgically or genetically modeled HD mice re-populated GABAergic MSNs neuronal loss in the striatum, and significantly improved cognitive and motor deficits. In a recent study, Schaffer and colleagues developed a scalable biomaterial-based 3D

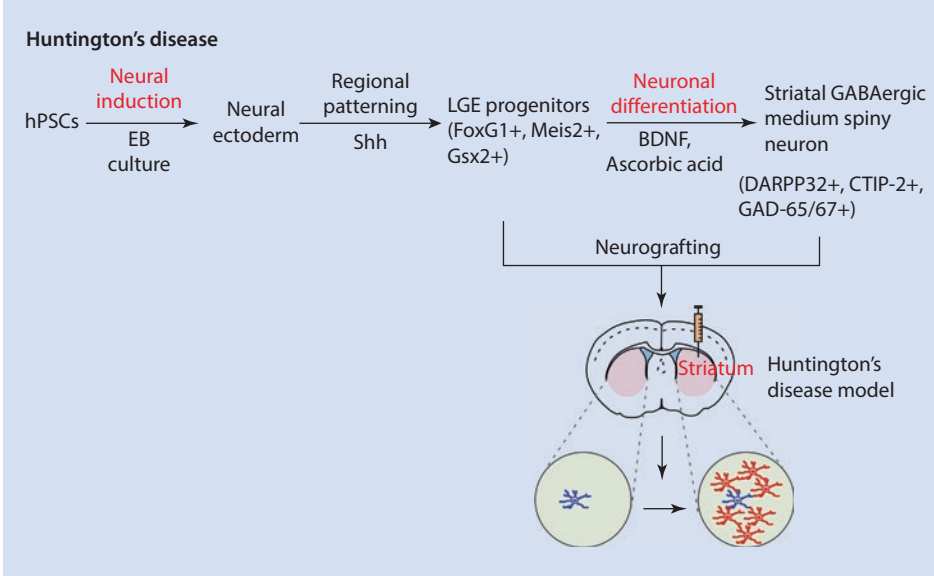

Fig. 9.4 Targeted LGE progenitor and MSN differentiation for cell replacement therapy for HD

platform to generate LGE progenitors and MSNs from hPSCs. LGE progenitors generated from this 3D system showed a better survival of transplanted cells and functional recovery in transgenic mouse HD model, suggesting the robustness of combined material science and stem cell techniques in cell-based replacement therapy (Fig. 9.4).

9.6 MGE Progenitor Differentiation and Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disease associated with serious loss of presynaptic cholinergic functions. AD patients usually suffer from a progressive decline in memory and cognitive function as well as behavioral symptoms, such as disorientation and hallucinations. Previous studies indicated that the decreased acetylcholine (ACh) release is a major feature of AD, which results from the declined number and functionality of basal forebrain cholinergic neurons (BFCNs) in a relatively early stage of AD. Several strategies exist in order to alleviate AD symptoms. For example, additional supply of neurotrophic factors (NTFs) directly or through a cell carrier is a way to improve the survival and function of cholinergic neurons. Acetylcholinesterase Inhibitor (AchEI) and N-methyl-D-aspartate receptor agonist treatment have also been applied in AD disease models or even patients, which show moderate benefits in cognition and memory recovery. However, all these therapeutic strategies only showed mild and temporary effectiveness.

Cell replacement therapy has also been proposed to be an ultimate way to cure AD. MGE is the sole origin to generate BFCNs during embryogenesis. Both hESCs and hiPSCs have been efficiently differentiated toward a MGE fate and then BFCNs (Liu et al. 2013; Yue et al. 2015). High concentrations of Shh under the EB differentiation conditions is the key to regionalize neural ectodermal cells to MGE progenitors, which belongs to the ventral most part of telencephalon. Blocking the Wnts pathway concomitantly with the

presence of the ventralization morphogen Shh will facilitate ventral patterning. Though Shh treatment in AD differentiated hPSCs will guide the cells otherwise to the FP fate, small molecules inhibiting the Wnts/P38/JAK-STAT pathways generates MGE progenitors under the AD differentiation conditions. The MGE progenitors differentiated *in vitro* uniformly express Nkx2.1 and FoxG1. Maturated BFCNs differentiated from these MGE progenitors express MAP2 and Synapsin, key functional neuronal markers, and ChAT, the rate limited enzyme for synthesize ACh. The *in vitro* produced BFCNs also showed active action potentials and synaptic activities over long periods of maturation in the culture. Both with surgical lesion and transgenic AD mouse models, transplantation of MGE progenitors into the bilateral hippocampus or basal nuclear shows clear and striking cognitive functional recovery. Histological and electrophysiological studies have also confirmed BFCN differentiation, maturation, long-term survival, and forming defined neural circuits with local neurons (Fig. 9.5).

9.7 FP Progenitor Differentiation and Parkinson's Disease

As one of the most prominent neurodegenerative disorders, Parkinson's disease (PD) bearing patients usually suffer from tremor, hypokinesia, rigidity and abnormal gait and posture. Pathological studies show that the cellular events underlying PD is the progressive death of dopaminergic (DA) neurons reside in the Substantia nigra, which causes insufficient release of dopamine in the striatum where DA neurons project.

The most commonly used drug for treating PD in the clinic is L-DOPA, which is selectively transported into the DA neurons and where it is readily converted to dopamine to compensate the reduced dopamine release in the caudate nucleus and putamen. L-DOPA has been observed with significant clinical benefit, but long-term use of L-DOPA showed ineffectiveness and has side effects, such as the on–off fluctuations and the emergence of dyskinesias. Therefore, there is a need for a more complete and long lasting method for restoring dopamine neurotransmission.

In 1992, human fetal mesencephalic tissues were transplanted into the caudate nucleus and putamen of Parkinson's patients for clinical trials. Symptoms, such as periods of dyskinesia and off episodes, were improved in patients after transplantation. This proof-of-concept clinical study suggests that cell transplantation therapy holds great potential for treating PD. However, several clinical studies failed to reveal statistically significant outcomes of fetal mesencephalic tissues for replacement therapy of PD, probably because of the variable quality of the donor tissues.

Midbrain DA neurons have now efficiently been generated from hPSCs (Kriks et al. 2011; Xi et al. 2012; Steinbeck et al. 2015; Wu et al. 2015; Chen et al. 2016; Kikuchi et al. 2017). It is now well acknowledged that the AD differentiation procedure is the most appropriate way to generate FP progenitors and DA neurons. Through applying Shh and FGF8 to ventralize and caudalize the adherently cultured hPSCs to induce a midbrain FP progenitor fate (EN1+/OTX2+ /FOXA2+/ LMX1A+) and adjusting Wnts signaling to promote dopaminergic differentiation, midbrain DA neurons (TH+/EN1+/OTX2+/FOXA2+/LMX1A+/NURR1+) can be efficiently produced. Moreover, these differentiated DA neurons displayed spontaneous action potential spikes, and this spiking was accompanied by a slow, subthreshold oscillatory potential resembling midbrain DA neurons *in vivo*. In addition, after transplantation of these midbrain DA neuronal progenitors into the striatum of the PD mouse or rat model, a complete restoration of amphetamine-

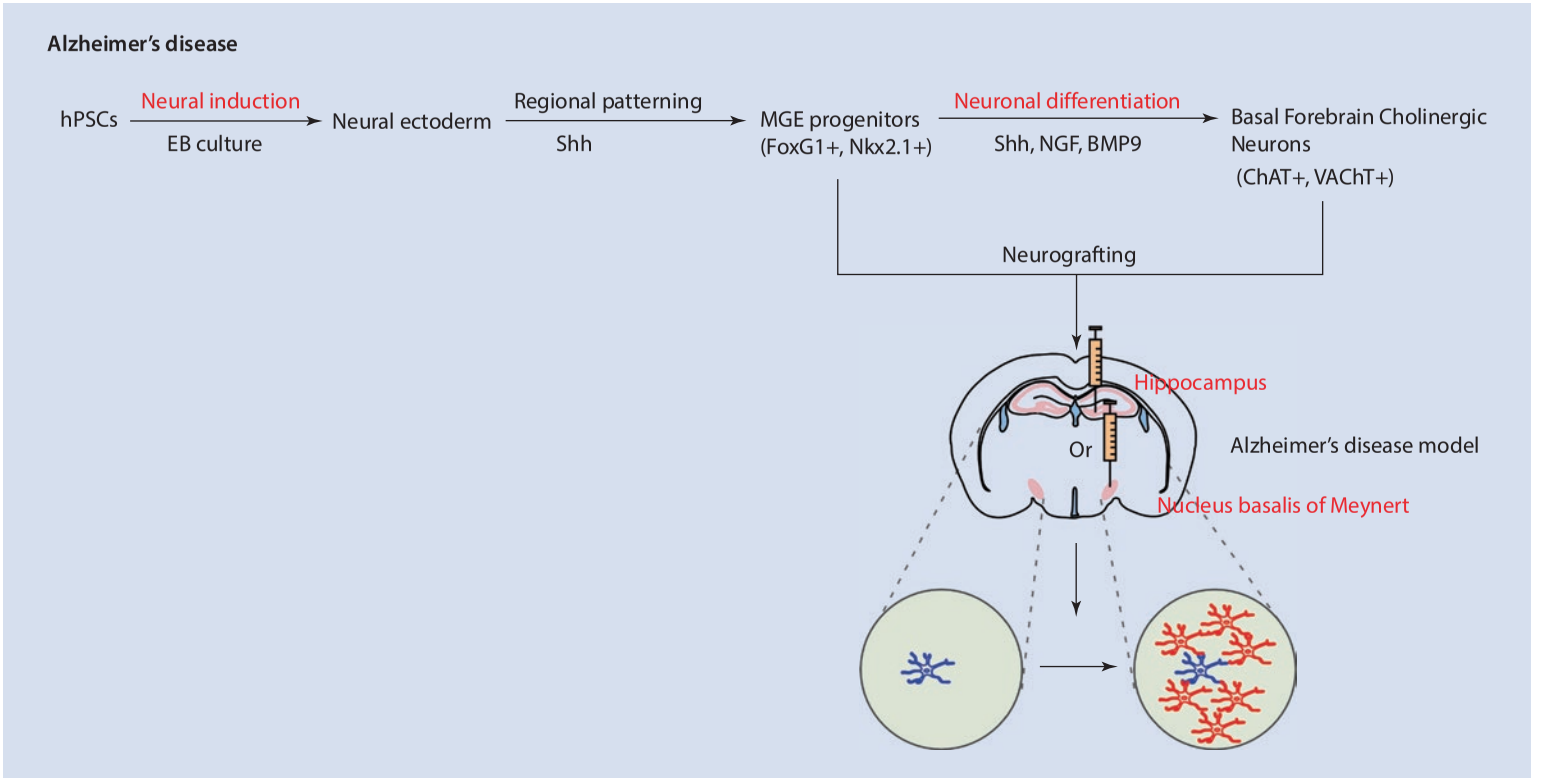

Fig. 9.5 Targeted MGE progenitor and BFCN differentiation for cell replacement therapy for AD

induced rotation behavior and improvements in tests of forelimb use and akinesia are observed. Notably, the efficacy of hPSCs-based dopaminergic progenitor cells transplantation in PD has now been also proved in monkeys and under clinical trials (Wang et al. 2018). A pioneering study conducted by Zhang and colleagues developed a system that enables precise regulation of hPSCs-derived neuronal activity for *in vivo* transplantation through chemogenetics (Chen et al. 2016). The midbrain DA neurons differentiated from hPSCs engineered to express DREADDs (designer receptors exclusively activated by designer drug) showed tight regulation of the activity of engrafted neurons, thus offering more accurate tools for future PD treatment (Fig. 9.6).

9.8 Conclusions and Perspectives

In this chapter, basic neural developmental concepts, especially related to neural induction and regional specification are introduced. Importantly, the basic neural developmental principles also apply to hPSCs when they are differentiated toward a neural fate. Both neural induction and regional patterning follows an activation/transformation model, that is, the cells will first take a cell fate by default and additive inductive signals are required to guide the cells to other fates instead. Both hESCs and hiPSCs hold great potentials in generating bona fide regional human NPs and according neuronal subtypes, which will offer unlimited cell sources for cell based replacement therapy for those publicly concerned neurodegenerative diseases, such as HD, AD and PD. Though exciting and promising evidence is accumulating by applying hPSCs in treating neurological diseases in animal models, careful and systematic clinical trials are urgently needed in order to fully prove the effectiveness and safety of applying hPSCs in treating patients. Integrating material science and genetic engineering techniques in cell replacement therapy in a plus to safeguard and move forward this promising approach to an ultimate success in the clinic.

Take Home Message

1. The entire process of neural development can be categorized into three stages in sequential, neural induction, regional patterning as well as neurogenesis and gliogenesis.
2. The activation/transformation paradigm applies to most of the cell fate determination events during *in vivo* neural development and *in vitro* neural differentiation.
3. Extracellular cues, such as TGFβ, BMPs, Wnts, Shh, RA and FGFs signaling molecules, are crucial cell fate inducers or blockers for either neural induction or regional patterning of neural progenitors.
4. Human pluripotent stem cells could be efficiently targeted to neural ectoderm cells, various regional neural progenitors, and different neuronal subtypes, which mirrors *in vivo* neural development.
5. A series of proof-of-concept laboratory studies have validated the efficacy of human pluripotent stem cell-based replacement therapy for currently incurable neurological diseases.

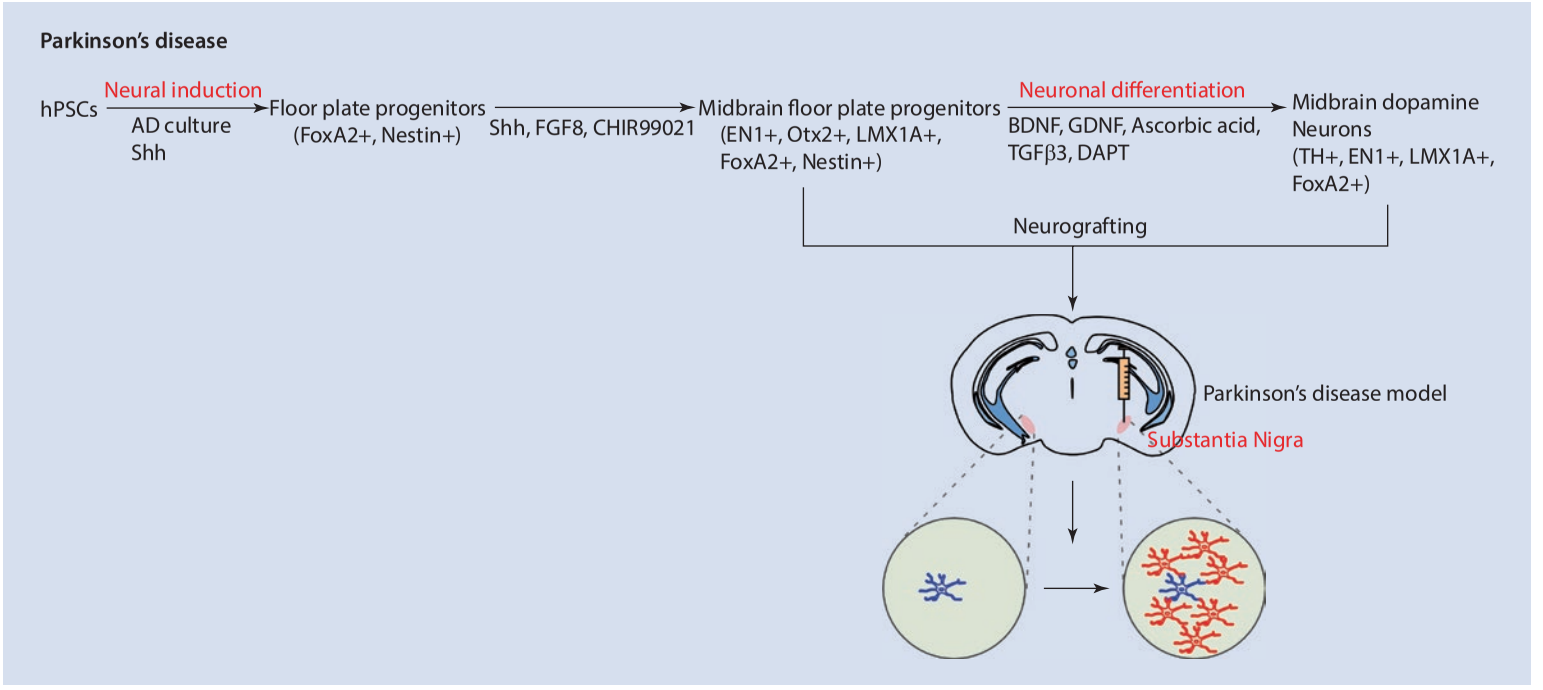

Fig. 9.6 Targeted midbrain FP progenitor and DA neuron differentiation for cell replacement therapy for PD

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