PROSPECT

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# Human ES and iPS Cells as Cell Sources for the Treatment of Parkinson's Disease: Current State and Problems

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## ABSTRACT

Cell therapy using human embryonic stem cells (hESCs) is a promising therapeutic option for Parkinson's disease (PD), an incurable neurodegenerative disease. A prerequisite for clinical application of hESCs for PD is an efficient and strict differentiation of hESCs into midbrain dopamine (mDA) neuron-like cells, which would be directly translated into high effectiveness of the therapy with minimum risk of undesirable side effects. Due to fruitful efforts from many laboratories, a variety of strategies for improving efficiency of dopaminergic differentiation from hESCs have been developed, mostly by optimizing culture conditions, genetic modification, and modulating intracellular signaling pathways. The rapid advances in the fields of dopaminergic differentiation of hESCs, prevention of tumor formation, and establishment of safe human induced pluripotent stem cells (hiPSCs) would open the door to highly effective, tumor-free, and immune rejection-free cell therapy for PD in the near future. J. Cell. Biochem. 109: 292–301, 2010. © 2009 Wiley-Liss, Inc.

**KEY WORDS:** HUMAN ES/IPS CELLS, DOPAMINE NEURON DIFFERENTIATION, PAKINSON'S DISEASE

Parkinson's disease (PD), which is known to affect about 1% of the population over 65 worldwide [Twelves et al., 2003], was first reported by Dr. James Parkinson in 1817. The cardinal symptoms of this devastating disease are resting tremor, rigidity and bradykinesia/akinesia [Agid, 1991; Lang and Lozano, 1998]. In addition, there are other symptoms such as depression, difficulty in swallowing, speech problems, sleep disturbances, fatigue, and autonomic disturbances, although the symptoms vary from patient to patient. PD is caused by preferential loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) of the ventral midbrain, leading to decrease in DA level in the striatum, mostly caudate and putamen areas, where the axons of SNpc DA neurons project [Ehringer and Hornykiewicz, 1960; Olanow, 2004]. The reduced DA level in the striatum is largely responsible for the motor dysfunctions seen in PD patients. It has been reported that the parkinsonian symptoms became apparent when 60–70% of the SNpc DA neurons die and 70–80% of dopaminergic nerve terminals are lost in the striatum [Bernheimer et al., 1973]. Unfortunately, there is no therapy available to cure PD. A commonly used pharmacological therapy using L-3,4-dihydroxyphenylalanine (L-DOPA), a dopamine precursor, is aimed to enhance DA level in the striatum by remaining DA nerve terminals. However, despite the initial symptomatic relief, this drug loses its efficacy as the disease progresses, and develops serious side effects after long-term use, such as "on–off" effect, dyskinesia and several psychiatric symptoms [Olanow, 2004]. A surgical treatment called deep brain stimulation (DBS) is also being used due to its efficacy in some PD patients. Stimulation of subthalamic nucleus or globus pallidus pars interna using electrical impulses was shown to ameliorate general parkinsonian symptoms [Limousin et al., 1998; Volkmann et al., 1998]. However, this approach is beneficial to only a population of PD patients and is an invasive therapy associate with potential risks, albeit rare, of intracranial hemorrhage, infection, seizure, pulmonary embolism, and psychiatric complications such as hallucination and depression [Umemura et al., 2003]. Although helpful, most of the current therapies only provide symptomatic relief and do not protect dying

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midbrain dopamine (mDA) neurons nor restore dead cell population. Therefore, cell replacement therapy has been emerging as a promising option for treating PD.

Since the first transplantation of human fetal mesencephalic tissue in 1987 [Brundin et al., 1987], many open-label trials have shown the efficacy of the fetal cell transplantation in PD patients. Importantly, the grafted DA neurons were found to survive over 10 years [Freed et al., 1992; Wenning et al., 1997; Piccini et al., 1999; Freed et al., 2001; Mendez et al., 2008]. However, two NIH-sponsored, double-blind clinical trials failed to show behavioral recoveries seen in the previous open-label trials [Freed et al., 2001; Olanow et al., 2003], which may be explained by multiple reasons such as no or short-term immune suppression, difference in tissue preparation methods, and problems associated with donor cells and patient selection. Furthermore, graft-induced dyskinesias were observed in a group of grafted patients [Freed et al., 2001]. Collectively, these results suggest that the fetal cell transplantation therapy may need optimal adjustment of its detailed procedure in order to make reproducible clinical outcomes. Nonetheless, a number of positive outcomes of fetal cell transplantation from several open-label trials strongly support the effectiveness of cell therapy to treat PD.

Although promising, the fetal cell transplantation therapy has some inherent limitations such as (1) short supply of donor tissues and (2) difficulty in standardization of the method and donor tissues for each trial. In this regard, human embryonic stem cells (hESCs) [and also human induced pluripotent stem cells (hiPSCs)] could be a potential future alternative cell source for cell therapy for PD. In this article, we summarized the current state of DA neuronal derivation in vitro from both hESCs and hiPSCs. Furthermore, behavioral outcomes of several transplantation studies in animal PD models were covered briefly. Lastly, current loadblocks that need to be removed for clinical applications of hESCs and hiPSCs to treat PD were discussed.

## hESCs AS AN INEXHAUSTIBLE SOURCE OF CELLS FOR CELL REPLACEMENT THERAPY

hESCs were first established by James Thomson in 1998 from the inner cell mass of a blastocyst [Thomson et al., 1998]. Due to their self-renewal and pluripotent characteristics, these cells have the capability of being expanded indefinitely and differentiated into any cell type of interest in appropriate conditions. In January 2009, the U.S. Food and Drug Administration (FDA) approved the first-ever hESC-based investigational new drug (IND) application to treat spinal cord injury (although it was put on hold several months later due to formation of microscopic cysts). In addition, several IND applications for hESC-based cell therapies are currently waiting for clearance from U.S. FDA.

Two decades of transplantation trials using human fetal mesencephalic tissue demonstrated that successful grafting of DA neurons into the striatum of the PD patients could lead to amelioration of DA-related motor dysfunctions. In that sense, cell therapy using hESCs is very promising as long as the issues of efficient derivation of DA neurons and safety are resolved.

## INITIAL STUDIES: DIFFERENTIATION OF hESCs INTO NEURAL PRECURSORS (NPs)

For clinical applications for PD, it would be critical to acquire pure population of mDA neuron-like cells from hESCs. This would obviate potential undesirable side effects derived from contaminated non-dopaminergic cells or remaining undifferentiated hESCs. Establishing a method for strict and efficient differentiation of hESCs into DA neurons is one of the processes required to obtain homogeneous DA neuronal population. To this end, coaxing hESCs efficiently into neural lineage, which is the first step of the whole dopaminergic differentiation procedure, should be achieved. In 2001, three groups separately reported protocols to generate NPs from hESCs [Carpenter et al., 2001; Reubinoff et al., 2001; Zhang et al., 2001]. Zhang et al. cultivated hESC-derived embryoid bodies (EBs) in suspension for 4 days, followed by culture the EBs in tissue culture dishes in basic fibroblast growth factor (bFGF)-supplemented medium until neural rosettes were formed. The neural rosettes which mainly consisted of nestin-positive NPs (96%) were isolated and further expanded in suspension as so-called "ESC-derived neurosphere" [Zhang et al., 2001]. Reubinoff et al. established a somewhat different method. They induced neural differentiation by overgrowing hESCs for 3–4 weeks, followed by expansion of the neural cells in suspension (as neural spheres) up to 22 weeks. The expanded spheres were mostly composed of NPs expressing nestin (97%), A2B5 (90%), and N-CAM (99%) [Reubinoff et al., 2001]. Carpenter et al. first generated EBs and then grew the cells on poly L-lysine/fibronectin-coated dishes in defined growth medium supplemented with epidermal growth factor (EGF), bFGF, platelet-derived growth factor-AA (PDGF-AA), and insulin-like growth factor-1 (IGF-1). The expanded cells were enriched by immunoselection for polysialylated neural adhesion molecule (PSA-NCAM) and A2B5, resulting in as much as 90% PSA-NCAM-positive or A2B5-positive NPs [Carpenter et al., 2001].

The NPs generated by these methods were able to be further differentiated into cell types of three neural lineages; neurons, astrocytes, and oligodendrocytes [Reubinoff et al., 2001; Zhang et al., 2001], and the NP-derived neurons were shown to display similar characteristics to mature neurons obtained from primary tissue [Carpenter et al., 2001].

## DIFFERENTIATION OF hESCs INTO CELLS WITH mDA NEURON-LIKE CHARACTERISTICS

### DOPAMINERGIC DIFFERENTIATION PROTOCOLS BASED ON TWO DIFFERENT NEURAL INDUCTION METHODS

Basic structures of protocols for differentiating hESCs into DA neurons have been established based on the information obtained from mouse embryonic stem cell (mESC) differentiation, which are largely categorized into two: (1) co-culture of ESCs with stromal feeder cells such as PA6 [Kawasaki et al., 2000] and (2) EB-based multi-stage method [Lee et al., 2000].

Co-cultivation with stromal feeder cells has been adopted by many groups for derivation of DA neurons from hESCs [Buytaert-

Hoefen et al., 2004; Park et al., 2004, 2005; Perrier et al., 2004; Zeng et al., 2004; Sonntag et al., 2007]. For example, Zeng et al. cultivated hESCs on PA6 stromal feeder cells for 3 weeks, which resulted in appearance of TH+ cells in about 87% of total colonies. These TH+ cells co-expressed other DA neuron markers, retained the capability of synthesis and release of dopamine, and formed a graft stably integrated into the striatum of 6-OHDA-lesioned PD rats [Zeng et al., 2004]. Perrier et al. used MS5 as stromal feeder cells to induce neural differentiation. The NPs obtained after 28 days of co-cultivation were first expanded on polyornithine/laminin-coated culture dishes in the presence of sonic hedgehog (SHH), fibroblast growth factor 8 (FGF8), brain-derived neurotrophic factor (BDNF) and ascorbic acid (AA), and then differentiated into DA neurons in N2 medium containing BDNF, glial cell line-derived neurotrophic factor (GDNF), TGFβ3, dibutyryl-cAMP (dbcAMP) and AA. This protocol yielded a high number of TH+ cells (about 30–50% of total cells were neurons and 64–79% of the neurons were TH+ cells) [Perrier et al., 2004].

EB-based method has been also used by many laboratories for differentiation of hESCs into DA neurons [Schulz et al., 2004; Yan et al., 2005; Iacovitti et al., 2007; Cho et al., 2008]. Schulz et al. performed differentiation of hESCs in suspension culture (as EBs) for about 1 month either in HepG2-conditioned medium supplemented with bFGF or in DMEM/N2 media containing serum and/or BMP4. After these relatively simple differentiation procedures, about 75% of total neurons (βIII-tubulin+) were found to be TH+ cells which expressed several DA neuronal markers, released dopamine upon stimulation, and formed stable grafts in PD rats [Schulz et al., 2004]. From a recent report, Roy et al. established an interesting protocol by combining EB-based method with co-culture method. In this protocol, EBs were formed first, and then fragmented and cultivated on tissue culture dishes to induce neural structures such as rosettes. The neural rosettes were collected, triturated and co-cultivated with immortalized human midbrain astrocytes to derive dopaminergic progenitors as well as DA neurons. After full dopaminergic differentiation, they were able to obtain a decent number of TH+ cells (about 39.6% of total cells were βIII-tubulin+ neurons and 67.4% of the neurons were TH+ cells) which were functional in terms of improving motor functions in PD rats after transplantation [Roy et al., 2006]. More recently, Cho et al. reported a highly efficient differentiation protocol. Like other methods, EBs were generated, fragmented and then induced to form neural structures (e.g., neural rosettes and neural tube-like structures). The most unique feature of this protocol is the generation of neurosphere-like structures, so-called "spherical neural masses" (SNMs), from the neural structures. The SNMs were passaged several times in suspension during which non-neural components were removed mechanically. These SNMs were able to produce high yield of functional TH+ cells after differentiation: 77% of total cells were neurons and 86% of the neurons were TH+ cells. Furthermore, the SNMs could be expanded for a long time, frozen and thawed freely on demand, and differentiated into DA neurons within only 14 days [Cho et al., 2008]. These characteristics would be of great help in producing a large number of DA neurons within a short period of time for cell transplantation.

## ENHANCING THE EFFICIENCY OF DOPAMINERGIC DIFFERENTIATION BY GENETIC MODIFICATIONS

Several attempts have been made to promote the efficiency of dopaminergic differentiation by genetically modifying hESCs. The genes used for the genetic modification were mostly, if not all, involved either in early development/specification of mDA neurons or in cell survival. To our expectations, these genes were found to support more efficient differentiation of hESCs into DA neurons as described below.

Martinat et al. tried to promote dopaminergic differentiation by lentiviral-mediated delivery of Pitx3 and Nurr1 genes, the two genes encoding transcription factors implicated in early mDA neuronal specification. Differentiation of the genetically modified hESCs was carried out by either co-cultivation with stromal cells or EB-based method. Both RT-PCR and immunocytochemistry analyses showed that overexpression of Nurr1 and Pitx3 genes increased the number of TH+ cells as well as the level of several DA neuronal markers after differentiation, but not the number of total neurons. Transplantation of the hESC-derived TH+ neurons was shown to elicit robust behavioral improvement in PD rats, albeit the number of animals per group in this study (n = 3–5) was small [Martinat et al., 2006].

Frilling et al. recently showed that exogenous expression of Lmx1a gene in hESCs led to a dramatic increase in the number of DA neurons. On the contrary, the number of serotonergic neurons was decreased. The genetically modified hESCs harboring the "PGK promoter-Lmx1a gene" cassette produced much higher TH+ cells (50–65% of total neurons) than control EGFP-hESC lines (25%). Importantly, the majority (75–85%) of TH+ cells from the Lmx1a-hESCs co-expressed several other DA neuron markers, while only a small portion of TH+ cells (10–20%) derived from EGFP-hESCs co-expressed the DA markers [Friling et al., 2009].

Ko et al. [2007] previously reported that hESC-derived NPs could be expanded over 20,000-fold without losing their capabilities of being differentiated into DA neurons. In their subsequent report, Ko et al. argued that the expanded hESC-derived NPs were prone to form tumors and undergo apoptosis. Intriguingly, overexpression of exogenous Bcl-XL and SHH enhanced viability of the hESC-derived NPs during differentiation in vitro as well as in vivo, leading to enhanced TH+ cell number (>1,000 TH+ cells) in graft and behavioral recovery in PD rats [Ko et al., 2009].

## ENHANCING THE EFFICIENCY OF DOPAMINERGIC DIFFERENTIATION BY MODULATING SIGNALING PATHWAYS

One interesting way to enhance efficiency of dopaminergic differentiation is by modulating signaling pathway(s) involved in neural induction and/or specification of mDA neurons. Based on the study that antagonistic inhibition of bone morphogenetic protein (BMP) signaling pathways promoted neural induction [Gerrard et al., 2005], Noggin, a peptide antagonist of BMP signals, has been included in many protocols [Iacovitti et al., 2007; Sonntag et al., 2007]. In addition, Chambers et al., demonstrated that blocking both BMP and TGFβ signaling pathways using Noggin and SB43154, respectively, resulted in much efficient neural induction [Chambers et al., 2009]. Other groups have shown that, treatment with signaling molecules such as Wnt5a and FGF-20 also enhanced dopaminergic

differentiation from hESCs [Correia et al., 2007; Hayashi et al., 2008; Shimada et al., 2009].

### DOPAMINERGIC DIFFERENTIATION USING INDUCED PLURIPOTENT STEM CELLS (iPSCs)

The groundbreaking discovery that overexpression of 4 transgenes, Oct4, Sox2, Klf4, and c-Myc, could reprogram differentiated somatic cells to the ESC-like pluripotent state [Takahashi and Yamanaka, 2006] opened a new avenue in cell replacement therapy. The somatic cell-derived pluripotent stem cells, termed iPSCs, could become an unlimited and autologous cell source that does not elicit immune rejection.

For clinical applications for PD, it would be crucial that hiPSCs can be efficiently differentiated into DA neurons and reverse behavioral defect in rat PD model. Although the feasibility of differentiating iPSCs into DA neurons was first demonstrated using hiPSCs [Takahashi et al., 2007], more extensive analyses were performed using mouse iPSCs. Wernig et al. reported that TH+ neurons were generated from mouse iPSCs in vitro, albeit with low efficiency (approximately 1–5% of total neurons). When transplanted, the mouse iPSC-derived neurons formed synaptic connections with host neurons and displayed behavioral recovery in PD rats [Wernig et al., 2008].

PD-specific hiPSCs were first established by Dr. Daley's group in 2008. In this case, the hiPSCs were generated from dermal fibroblasts of 57-year-old male suffering from multifactorial PD [Park et al., 2008]. The disease-specific hiPSCs would provide a useful tool for studying etiology of the disease, toxicological research, and drug-screening. Soldner et al. also successfully established hiPSC lines from seven different PD patients and were able to differentiate the PD-specific hiPSCs into DA neurons using both EB-based and co-culture methods. Interestingly, no difference in the efficiency of dopaminergic differentiation was detected among the PD-specific hiPSCs, non-PD-hiPSCs and hESCs (about 2–4% TH+ cells/total cells and 10–45% neurons/total cells) [Soldner et al., 2009]. However, this result needs further confirmation since the condition for dopaminergic differentiation used in this report seemed to be suboptimal as judged by low efficiency of DA neuronal generation even from hESCs. Furthermore, behavioral recovery of PD animal models by the iPSC-derived DA neurons was not examined in this report.

In summary, hiPSCs derived from both normal adults as well as PD patients were shown to have the capability to be differentiated into DA neurons. However, both efficient differentiation of hiPSCs into DA neurons and behavioral functionality of the hiPSC-derived DA neurons in animal PD model are yet to be demonstrated.

## CRITICAL ISSUES TO CONSIDER FOR CLINICAL APPLICATIONS OF hESCs TO TREAT PD

Although great advancement has been made in dopaminergic differentiation of hESCs (Table I), many loadblocks still have to be removed before safe and successful use of hESCs to treat PD.

### GENERATION OF HOMOGENEOUS POPULATION OF mDA-NEURON LIKE CELLS FROM hESCs IN LARGE AMOUNTS

One of the critical issues to address before clinical applications of hESCs for PD is obtaining pure population of DA neurons or DA neural precursors. Contamination of even small number of undifferentiated hESCs in the graft could be a potential source for tumor formation. It should be also noted that the presence of other types of cells could cause undesirable side effects. For example, a recent report demonstrated that serotonin neurons existing in the graft worsened L-DOPA-induced dyskinesias in PD rats, probably by the release of L-DOPA-derived DA from serotonin nerve terminals [Carlsson et al., 2007].

Two technical improvements may contribute to obtaining a pure population of DA neuronal precursors or DA neurons; (1) highly efficient and strict differentiation method, and (2) sorting the DA neuronal precursors or DA neurons. Although diligent efforts from many laboratories have led to higher yield of DA neurons as shown in Table I, there still remains much room for improvement in dopaminergic differentiation of hESCs. Until now, the most efficient protocol for DA neuronal generation from hESCs in the absence of genetic modification yielded 86% of DA neurons over total neurons and 77% of neurons (βIII-tubulin+) out of total cells in vitro [Cho et al., 2008]. Although genetic modification could enhance the efficiency of dopaminergic differentiation [Friling et al., 2009], this approach may need extra caution to use it for clinical applications due to risks of undesirable side effects.

Cell sorting is another way to obtain a homogeneous population of DA neurons. For example, several groups tried to isolate DA neurons by sorting genetically manipulated hESCs where EGFP was expressed by either Pitx3 or dopamine transporter gene promoter [Hedlund et al., 2008; Zhou et al., 2009]. Other groups tried to remove only remnant undifferentiated hESCs from differentiated cell population by sorting the cells with antibody against PSA-NCAM [Schmandt et al., 2005; Thompson et al., 2006]. In this case, non-dopaminergic cells are still remaining in the cell population, posing the risk of unwanted side effects.

Ideally, it would be better if mDA neuron-specific cell surface marker is available for cell sorting. Corin might be one of the candidates which fall into this category [Jonsson et al., 2009].

### ACHIEVING GOOD SURVIVAL OF hESC-DERIVED CELLS AFTER TRANSPLANTATION

Good survival of the transplanted cells is an important issue to consider for obtaining maximal clinical benefits from future cell therapy for PD. Since more than 200,000 DA neurons should survive for behavioral restoration [Hagell and Brundin, 2001], both efficient generation of DA neurons and maximal survival of DA neurons after transplantation are absolutely required for a successful cell transplantation therapy. Many reports so far showed that the number of DA neurons survived in the graft of PD rats was very low compared with the original number of cells used for transplantation (about 200,000–500,000 cells). Therefore, optimal conditions for an effective transplantation need to be determined carefully. Examples of strategies to enhance survival of grafted DA neurons are described below:

TABLE I. Previous Studies on the Differentiation of hESCs Into DA Neurons

| Differentiation method | Length | Differentiation efficiency in vitro | Behavioral efficacy | References |
|---|---|---|---|---|
| Co-culture with PA6 | ~3 Weeks | 87% of colonies contain TH+cells | ND | Zeng et al. [2004] |
| Co-culture with astrocytes derived from rat embryonic striatum<br>Co-culture with astrocytes derived from rat embryonic mesencephalon<br>Co-culture on PA6 in the absence or presence of GDNF | ~3–4 weeks<br>On embryonic mesencephalon: 33 TH+/well<br>PA6: 443 TH+/well<br>PA6 + GDNF: 934 TH+/well | On embryonic striatum: 329 TH+/well in 12well plate | ND | Buytaert-Hoefen et al. [2004] |
| Serum-free suspension culture in DMEM/F12/N2/MedII/fFGF2 (or in DMEM/F12/N2/MedII/FGF2 followed by in DMEM/N2) | >1 month | 75% TH+cells/bIII-tubulin+cells | ND | Schulz et al. [2004] |
| Grow hESCs on MEF in DMEM/10% FBS/Noggin for 8 days, followed by in DMEM/10% FBS for 6 days. Mechanically dissected neural progenitors were grown for 6 weeks as spheres in DMEM/F12/B27/EGFG/bFGF | >8 weeks | 0.56% TH+cells/bIII-tubulin+cells in vitro<br>0.18% TH+cells/total cells in vivo | Effective in four tests<br>Apomorphine-induced rotation<br>Amphetamine-induced rotation<br>Stepping adjustments<br>Forelimb placing test | Ben-Hur et al. [2004] |
| Neural induction for 4 weeks on MS5-based stromal feeder cells, followed by neural patterning by two passage-culture in N2 medium/SHH/FGF8/BDNF/AA for total 14–18 days. Finally dopaminergic differentiation was induced in N2 medium/BDNF/GDNF/TGFb3/dbcAMP/AA for more than 8 days | >50 days | 64–79% TH+cells/bIII-tubulin+cells<br>30–50% bIII-tubulin +cells/total cells | ND | Perrier et al. [2004] |
| Neural induction for ~10 days as EBs in suspension, followed by induction of midbrain specification with FGF8/SHH for 8 days. Full dopaminergic differentiation was induced in the presence of FGF8/SHH over 3 weeks | >38 days | 50–60% TH+cells/bIII-tubulin+cells<br>31.8% TH+cells/total cells | ND | Yan et al. [2005] |
| hESCs were differentiated into DA neurons by co-cultivation with bone marrow-derived stromal cells, followed by culture in defined medium without stromal cells. When NPs were formed during this procedure, Nurr1 and Pitx3 genes were introduced into the cells by lentiviral vector-mediated gene delivery | ~6 weeks | hESCs: ~1 TH+cells/field<br>hESC-Nurr1: ~4 TH+cells/field<br>hESCs Pitx3: ~1 bIII-tubulin+cells/field<br>hESC-Nurrl/Pitx3: ~8 bIII-tubulin+cells/field | Effective in<br>Apomorphine-Induced rotation (using 6-OHDA lesioned mice) | Martinet et al. [2006] |
| Co-culture with PA6 for a week in ITS/AA-medium, followed by culturing on a fresh PA6 in SHH/FGF8-containing medium for 1 more week. The resulting NPs were expanded on FN-coated dishes in N2/AA/bFGF-medium, followed by differentiation in ITS- and AA-containing medium | ~28 days | 95% Nestin+ or bIII-tubulin+/total cells<br>41%TH+/bIII-tubulin+cells | Not effective in:<br>Amphetamine-induced rotation<br>Step-adjustment test | Park et al. [2005] |
| Expansion of NPs by multiple passages on FN-coated plates in ITS/AA/bFGF-containing medium. Terminal differentiation was induced in the medium containing ITS/AA/BDNF/GDNF/dbcAMP | | 34–45% TH+/bIII-tubulin+cells | Effective in:<br>Amphetamine-induced rotation | Ko et al. [2007] |

| | | | | |
|---|---|---|---|---|
| Co-culture on MS5-wnt1 for 3 weeks, first in DMEN/KOSR/Noggin for 2 weeks, followed by 1-week culture in DMEM/F12/N2/Noggin. Neural rosettes were collected and expanded for 9 days on polyomithine/laminin-coated dishes in N2/BDNF/AA/SHH/FGF8/bFGF medium. Differentiation was induced for 12 days in N2/BDNF/AA/dbcAMP/GDNF/TGFb3 medium | ~49 days | 22.3% TH+cells/total cells | Three out of 12 grafted PD rats were effective in: Amphetamine-induced rotation On average, 160 TH+cells/rat (grafted with 100,000 cells) | Sonntag et al. [2007] |
| EBs cultured in suspension were plated on culture dishes for 10 day-culture to induce neural rosettes. Neural rosettes were triturated and grew with immortalized human midbrain astrocytes in DMEM/F12/N2/bFGF/laminin/SHH/FGF8 for 8 days and then in DMEM/F12/N2/laminin in the presence of immortalized human midbrain astrocytes for another 14 days | ~36 days | 39.6% bIII-tubulin+/total cells 67.4% TH+/bIII-tubulin+cells | Effective in three tests Apomorphine-induced rotation Adjusting step test Cylinder test | Roy et al. [2006] |
| Neural induction was initiated from EBs on collagen-coated dishes in defined media supplemented with fibronectin, noggin for 4 days. Neural structures formed were expanded on fresh collagen-coated dishes in the presence of bFGF. Final dopaminergic differentiation step was done in polyomithine-coated dishes for a week with dbcAMP treatment | ~21 days | 56–81% TH+/bIII-tubulin+cells | ND | Iacovitti et al. [2007] |
| NPs were induced from the EBs attached on Matrigel for 5 days and expanded for 4 days in an expansion medium containing bFGF and N2 supplement. Neural structures were mechanically isolated and grow as sphere ("SNM") for 7–10 days for expansion. The SNM can be expanded for a long time and differentiated into DA neurons in differentiation media supplemented with SHH, FGF8, and AA | ~40 days from hESCs ~14 days from SNMs | 86% TH+/bIII-tubulin+cells 77% bIII-tubulin+/total cells | Effective in three tests Apomorphine-induced rotation Amphetamine-induced rotation Adjusting step test | Cho et al. [2008] |
| Co-culture with human amniotic membrane matrix | >6 weeks | 39% TH+cells/bIII-tubulin+cells in vitro | ND | Ueno et al. [2006] |
| EBs were farmed, followed by selection of neural precursors in adherent culture. Neural cells were expanded with SHH and FGF8 and differentiated into the mDA neurons in neurobasal medium supplemented with IL-1beta and GDNF | ~50 days | Most of the cells become neurons 40% TH+cells/bIII-tubulin+cells | Effective in: Apomorphine-induced rotation over 1 year | Geeta et al. [2008] |

ND, not determined.

(1) Transplantation of cells in early stage of differentiation would lead to more heterogeneous cell population in the grafts, while grafting fully matured DA neurons with long branching processes may yield decreased cell survival due to higher vulnerability to injury.
(2) Efforts to alleviate cell stresses resulting from the change in growth environment during transplantation may increase cell survival in the grafts. For example, pretreating the cells with neurotrophic factors such as GDNF, BDNF and neurotrophin-3, prior to transplantation were shown to enhance cell survival [Apostolides et al., 1998; Espejo et al., 2000; Hoglinger et al., 2001; Andereggen et al., 2009].
(3) In addition, strategies for blocking apoptotic pathways, avoiding excitotoxicity, and inducing blood vessel formation near the grafted sites were also discussed to increase cell survival [Sortwell, 2003].

### PREVENTION OF TUMOR FORMATION AFTER TRANSPLANTATION

Tumor formation is one of the biggest loadblocks in taking hESCs into clinical trials. Several causes may be responsible for the tumor formation, which include (1) undifferentiated hESCs still existing in the cell population after dopaminergic differentiation, (2) chromosomal aberration in hESCs, and (3) co-existing of actively proliferating irrelevant cells in differentiated cell population. Therefore, to minimize the tumor formation, it would be critical to perform dopaminergic differentiation using hESCs with normal karyotype. Furthermore, establishing methods to remove hESCs or unwanted proliferating cells completely from the differentiated cell population either by strict dopaminergic differentiation or sorting of DA neurons (or DA neuronal precursors) are also required.

### PREVENTION OF IMMUNE REJECTION AFTER TRANSPLANTATION

Although brain is an immunologically privileged area, immune suppressant drug might be needed to prevent immune rejection of the graft by host immune system.

The iPSC research is expected to open new avenues in immune rejection-free and patient-specific cell therapy for PD. Recent success in generation of safe hiPSCs either by non-viral and chromosome non-integrating gene delivery system [Yu et al., 2009] or by protein delivery [Kim et al., 2009] would bring the hiPSCs one step closer to clinical applications. One of the remaining technical issues to be resolved, however, may be the generation of the hiPSCs in a defined and xeno-free culture condition.

### PROVISION OF LARGE AMOUNT OF DA NEURONS WITHIN A SHORT PERIOD OF TIME

It is estimated that more than 200,000 DA neurons per brain are needed for behavioral recovery [Hagell and Brundin, 2001]. Considering the estimated low cell survival rate after transplantation (5–20%) [Sortwell, 2003], a large number of DA neurons would be needed for cell transplantation. In this regards, development of protocols for enhancing both dopaminergic differentiation and expansion of NPs (or DA neuronal progenitors) would be of great importance. Furthermore, it would be more beneficial if the differentiation process becomes simpler and shorter.

To address this issue, massive expansion of hESC-derived NPs, which are easily differentiated into DA neurons on demand, may be an attractive idea. Recently, Ko et al. [2007] successfully expanded NPs over 1 month (>20,000-folds increase after six passages), and were able to differentiate the expanded NPs into DA neurons efficiently. Cho et al. [2008] also established a procedure in which spherical neural masses (SNMs), which are mostly composed of NPs, could be expanded for a long time (at least 120 days) and be coaxed with high efficiency into DA neurons within only 2 weeks. Similar approaches of expanding NPs were also shown in other reports [Geeta et al., 2008].

### GENERATION OF CLINICALLY COMPLIANT DA NEURONS FROM hESC

For clinical applications, it would be required to eliminate risk of the xenogenic contamination. First of all, hESCs for dopaminergic differentiation should be generated and cultivated in a xenogen-free environment. In fact, several studies have already reported successful derivation and maintenance of hESCs on either human feeder cells or feeder-free extracellular matrices [Xu et al., 2001; Ludwig et al., 2006]. Second, the whole dopaminergic differentiation procedure should be carried out under xenogen-free conditions. To this end, both culture media and feeder cells (or matrices in the case of feeder-free culture) should be carefully chosen. Cell culture medium should not contain any component from animal source. For example, fetal bovine serum should not be included in the medium. All the reagents, growth factors, and extracellular matrix should be clinical grade synthesized under good manufacturing practice (GMP) conditions. When co-culture method is to be adopted for the differentiation, mouse stromal cell lines such as MS5 and PA6 have to be replaced with human cell lines with similar functions. For example, immortalized human fetal mesencephalic astrocytes [Roy et al., 2006] were used for dopaminergic differentiation. Replacement of mouse feeder cells with feeder-free, non-cellular materials can be another option. Ueno et al. [2006] demonstrated that human amniotic membrane matrix, a non-cellular tissue material, had dual functions in differentiating hESCs into DA neurons: those functions were (1) neural cell formation and (2) dopaminergic specification. On the contrary, matrigel, one of the most popular extracellular matrices commercially available, should not be used since it is derived from mouse tumor cells.

In addition, it should be also noted that the fate determination of hESC-derived NPs could be affected by the nature of extracellular matrices used. For example, it was reported that poly-L-ornithine/laminin tends to induce neural cell formation, while plastic, gelatin and fibronectin favor nonneural cell formation [Goetz et al., 2006].

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

hESCs, which can supply unlimited number of cells for transplantation, are regarded as a promising cell source for cell therapy for PD. However, safety issues, especially immune rejection and tumor formation, need to be clearly resolved before clinical applications of the cells. The recent advent of iPSC research field is expected to provide a solution for the immune rejection problem. Establishment

of clinically compliant hiPSCs for a single patient may sound impractical at this moment. However, it is probable that the generation of patient-specific hiPSCs would become much easier and less labor-intensive in the near future due to rapid advances in the technical aspects of iPSC research [Aasen et al., 2008; Sun et al., 2009; Utikal et al., 2009]. Although the risk of tumor formation might not be easy to eliminate completely, development of strict and efficient dopaminergic differentiation protocols, sorting of the DA (or DA precursor) cells, and selective killing of the remnant hESCs in the cell population after differentiation would eventually lead to safe clinical applications of hESCs to treat PD.

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