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REVIEW ARTICLE

Application of Embryonic Stem Cells on Parkinson's Disease Therapy

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Embryonic stem (ES) cells have the ability to reproduce themselves for a long period and differentiate into specific morphological and functional cells. The ES cells are an important material in developmental biology, genomics, and transgenic methods, as well as in potential clinical applications, gene therapy and tissue engineering. The pluripotent ES cells will be a valuable source in the treatment of numerous functional degenerative pathologies including Parkinson's disease (PD), which is characterized by progressive and selective loss of dopaminergic neurons in the midbrain substantia nigra. Thus, the most important for using ES cells in PD therapy is to direct their differentiation into dopaminergic phenotype to replace the degenerated cells. In this review, we summarize the neural differentiation directing protocol, transplantation results, and behavioral recovery of ES cells derived dopaminergic neurons in the therapeutic studies in PD animal models.

Key Words: embryonic stem cells; Parkinson's disease

Introduction

Mammalian stem cells have the ability to prolongate proliferation and differentiate into several specialized cells that make up the tissues and organs of the body. Stem cells are classified by their sources into three classes: the embryonic stem (ES) cells that are derived from the inner cell mass of preimplantation blastocyst,[1–3] the embryonic germ cells that are derived from the primordial germ cells of the primitive genital ridge in the fetus,[4,5] and the adult stem cells that are isolated from differentiated tissues such as bone marrow, brain, blood, liver, pancreas, and skin.[6–8] The ES cells are believed to be most pluripotent, and under certain specific conditions, ES cells can give rise into cells of all three primary germ layers, ectoderm, mesoderm, and endoderm. Research in this area opens a new window into the embryonic developmental biology.[9] Consequently, the pluripotency of ES cells will be a valuable source of cell replacement therapy in numerous pathologies and has the potential to be used in cell transplantation and biomedical engineering.[10,11]

Many mammalian ES cells were derived in last 3 decades, including mouse,[1] human,[3] rabbit,[12] rat,[13] rhesus monkey,[14] marmoset,[15] pig,[16,17] and some presumptive pluripotent ES cells, including hamster,[18] sheep,[19] cattle,[20] and mink.[21] Research in establishing committed tissue-specific progenitors from ES cells and evidence of their function after

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transplantation in various animal disease models are also reviewed.[22] The ES cells can serve as important tools in developmental biology, genomics, and transgenic methods, as well as in potential clinical applications such as degenerative medicine, traumatic disorder, gene therapy and tissue engineering.[23,24]

Differences Between Human, Porcine and Murine ES Cells

Research has had remarkable results in inducing human ES cells differentiation into neuronal cells,[25,26] pancreatic β cells,[27] and cardiomyocytes.[28] These results reveal the potential for the clinical application of human ES cells for the treatment of diseases such as spinal cord injury,[29] Parkinson's disease (PD),[30] diabetes,[31] and heart diseases.[32] Although human ES cells hold a promising future for treating degenerative disorders such as Parkinson's and Alzheimer disease, or in treating spinal cord injury, the preclinical research must be proven in suitable animal models to present its bio-safety and long-term efficiency of transplanted ES cells and/or their derived cells. Studies demonstrate the existence of differences between human ES and murine ES cells, suggesting that the information gained from murine ES cells, especially the gene expression profiles during neural differentiation, cannot be directly translated to human ES cells.[17,33] Swine has been demonstrated as an excellent animal model in therapeutics development for various human diseases, including congenital heart disease,[34] hypertension,[35] organ transplantation,[36,37] pharmacology, and toxicology.[38,39] In addition, the colony morphology of the porcine ES cells are very similar to human ES, and they are both feeder-dependent and refractory to leukemia inhibit factor. Porcine ES cells cluster into colonies sharing a flattened morphology of human ES but not mouse ES cells.[16] They both express octamer-binding transcription factor-4 (Oct-4), alkaline phosphatase (AP), stage specific embryonic antigen-3/4 (SSEA-3/4), tumor related antigen (TRA) 1-60, and TRA 1-81, but not the SSEA1 that is characterized by mouse ES cells.[40] Therefore, the porcine ES cells can provide an optimal preclinical animal model in the study of regenerative medicine in therapeutic approaches.

Parkinson's Disease and Animal Research Models

PD is a degenerative disorder that was first described by Dr. James Parkinson in 1817. The PD patients show shaking rigidity, balance problems and slowed movement in behavioral symptoms. Several major medical approaches of PD therapy, including pharmacological therapy with the dopamine precursors such as L-3,4-dihydroxyphenylalanine (L-dopa), Carbidopa-Levodopa (Sinemet) and Selegiline hydrochloride (Eldepryl). This DA-replacement treatment takes effects during early stages but is associated with devastating side effects, such as dyskenesia.[41] Further more, implanted Deep Brain Stimulation in subthalamic nucleus to stimulate with tiny electrical current can reduce the abnormal movement of PD patient.[42] Another surgical approach is to transplant fetal ventral mesencephalic cells in PD patients.[43] However, fetal cells transplantation has limitations with technical and ethical issues.[44] Due to the self-renewal capacity and multilineage developmental potential, ES cells can be ideal cell sources for cell replacement therapy.[45,46]

For the ES cells transplantation and functional recovery studies of PD, the nigrostriatal lesion and amphetamine-induced rotation behavioral analyses are well developed. The 6-hydroxy dopamine (6-OHDA) is injected into the medial forebrain bundle (nigrostriatal dopaminergic pathway) with the assistance of a stereotaxic instrument (anterior-posterior, AP=−4.4 mm; medial-lateral, ML=−1.2 mm; dorsal-ventral, DV=−7.8 mm; and AP=−4.0 mm, ML=−0.8 mm, DV=−8.0 mm).[47,48] After lesioning, the animals demonstrate asymmetric rotations by using an automated rotometer (TSE, Germany)[49,50] in response to D-amphetamine challenge are characterized as well-lesioned PD models and are used for ES cells transplantation into the striatum (AP=0.0 mm, ML=−3.0 mm, DV=−5.0 mm).[48]

Neural Induction of ES cells

The efficiency for neural population conversion is the most important for stem cell replacement therapy in the field of neurological disorders. Although the low efficiency of neural differentiation derived from ES cells has been one of the major obstacles to overcome,[51] a significant improvement of neural lineages induction has been achieved by application of several morphogens such as *all-trans* retinoic acid (RA), sonic hedgehog (SHH), fibroblast growth factor (FGF), epidermal growth factor (EGF), bone morphogentic proteins (BMPs), and glial-derived neurotrophic factor (GDNF).[52–58] These molecules are essential for normal embryonic development and differentiation,[59] and are used as neurogenic stimulators of ES cells.[26,55,60] Perrier et al[57] develops an *in vitro* neural induction protocol of hES-derived dopaminergic differentiation by co-culture with stromal cells MS5 and S2. A similar protocol also reports using co-culture with PA6 stromal cells[61] and PA6 cells overexpressing PA6-SHH.[62] A rapid protocol

using the chemically defined culture system also allows human ES cells to differentiate into DA neurons by the addition of SHH and FGF-8,[63] or the recombinant human noggin, bFGF and dibutyryl-cAMP,[64] or FGF8b and SHH.[65] Those derived cells exhibit the specific dopaminergic markers such as Lmx-1b (a transcription factor regulated the development of midbrain DA neurons), Nurr-1 and Ptx-3 (transcription factors expressed in midbrain DA neurons), Girk2 (a potassium channel expressed specific to DA neurons in the substantia nigra) and TH (a biosynthetic enzyme of DA).[62,64–66] These successful induction protocols provide powerful tools to control the development and function of ES-derived midbrian DA neurons and makes ES cell therapy on PD promising in the future.

Porcine ES Cells in PD Therapy

In our laboratory, we establish a porcine ES cell line M215-3 derived from the blastocyst of the Livestock Research Institute Black Pig No. 1 (a topcrossing breed established from Taoyuan and Duroc pigs) (Figure 1); we also derived three GFP-expressing ES cell lines from M215-3 by electroporation designated as pES/GFP$^+$ 10, 14-1, and 14-2 (Figure 2).[40] They all maintain normal karyotype of 36+XX and express the pluripotent markers Oct-4, AP, SSEA-3, SSEA-4, TRA 1-60, and TRA 1-81. For the study on ES cell therapy in neural regenerative disorders, we develop a directed differentiation protocol including a neural induction culture for 12 days and extending culture for 6 days. This induction allows us to attain more differentiated neural lineages that also provide a valuable yield on the process of differentiation of porcine ES cells into functional neurons (Figure 3). Furthermore, the pigs offered some distinct advantages over other species and served as a better research model because they were immunologically and physiologically more similar to humans. This directed induction protocol will be helpful in further application of porcine ES cells in replacement medicine and in numerous functional degenerative pathologies. In our studies, we presented an *in situ* monitoring of these grafted cells with the assistance of a stereotaxic instrument, and their survival and development was determined every 15 days by two of live animal fluorescence optical imaging systems: the In Vivo Imaging System (IVIS 50; Xenogen Corp., Alameda, CA) was for the noninvasive tracking (Figure 4), and the real-time images of grafted cells in recipient's brain was performed by *ex vivo* fibered confocal Cellvizio Imaging System (Cellvizio, Mauna Kea Technologies, Cambridge, MA,

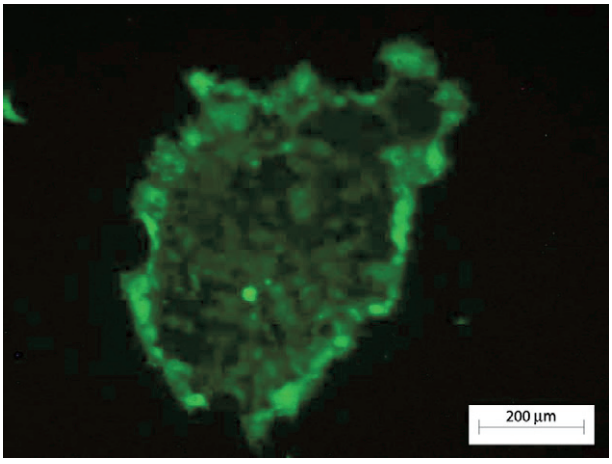

Figure 2 The colony morphology of porcine embryonic stem cells expressing green fluorescent protein (pES/GFP$^+$ 10).

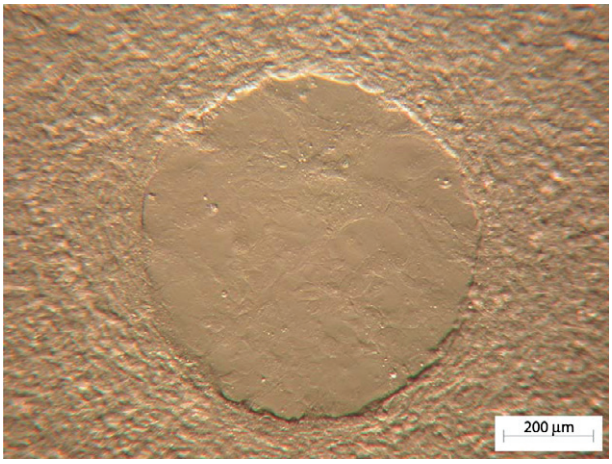

Figure 1 The colony morphology of porcine embryonic stem cells M-215-3.

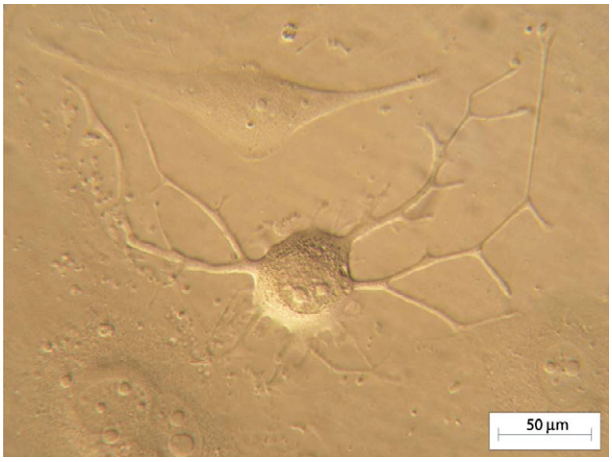

Figure 3 The porcine embryonic stem cell-derived neural cells showed a typical neural morphology with cell body and axons after directed differentiation.

USA) for invasive tracking during the 3 months of experimental period (Figure 5). We also injected pES/GFP$^+$ cells and their derived cells of neural differentiation stages into the 6-OHDA lesioned PD rats. The *in vivo* cell growth and differentiation of the grafted cells, and the functional recovery of the PD animals were investigated in our study.[48]

However, a major concern of ES transplantation is the tumorgenic potential if ES cells are to be applied clinically.[67] Consequently, the effort to determine those ES-differentiated cells posttransplantation *in vivo* aimed at monitoring the survival and development of grafted ES or ES-derived cells in PD therapy. The need for noninvasive or invasive monitoring of model systems has resulted in the development of small animal imaging technologies such as microcomputed tomography, micropositron emission tomography, magnetic resonance imaging,[68,69] or fluorescence cystoscopy with hexyl aminolevulinate,[70] optical coherence tomography,[71] positron emission tomography,[72] and fibered confocal microscopy.[73] All of which are now capable of providing real-time images and obtaining the fate of ES cells for therapeutic purposes,[72,74,75] the course of cancer metastasis and therapy,[69,76,77] and embryo development.[78]

To investigate the therapeutic potential in PD, we transplanted porcine ES cells into the Sprague-Dawley rat brain with the assistance of a stereotaxic instrument. Following 3 month of behavioral analyses after porcine ES cells transplantation, the functional behavior recovery analysis by amphetamine-induced rotation test indicated the grafted porcine ES-derived neuronal progenitors showed a decreased relative rotation rate and resulted in a functional recovery from PD behavioral defects.[48] The results of our study and several previous studies on PD therapy in the animal models are summarized in Table 1.[45,46,48,61,62,65,67,72,79–92] Several studies indicate that the grafting of mouse or human ES-derived DA neurons can reduce functional deficits significantly in PD animal models.[45,46,48,79,80–86] However, some studies show partial recovery from

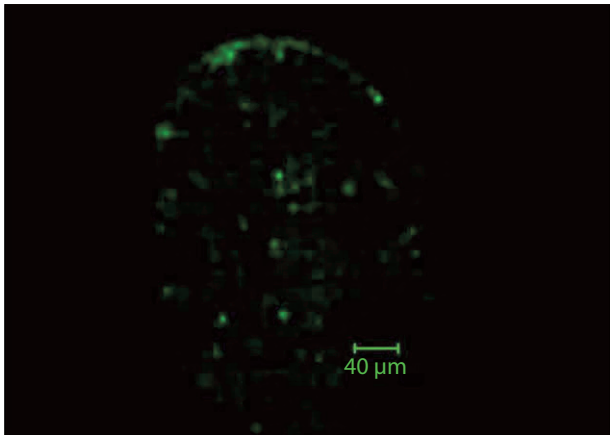

Figure 5 The real-time image of grafted pES/GFP$^+$ cells in recipient's brain was determined by using *ex vivo* fibered confocal Cellvizio Imaging System.

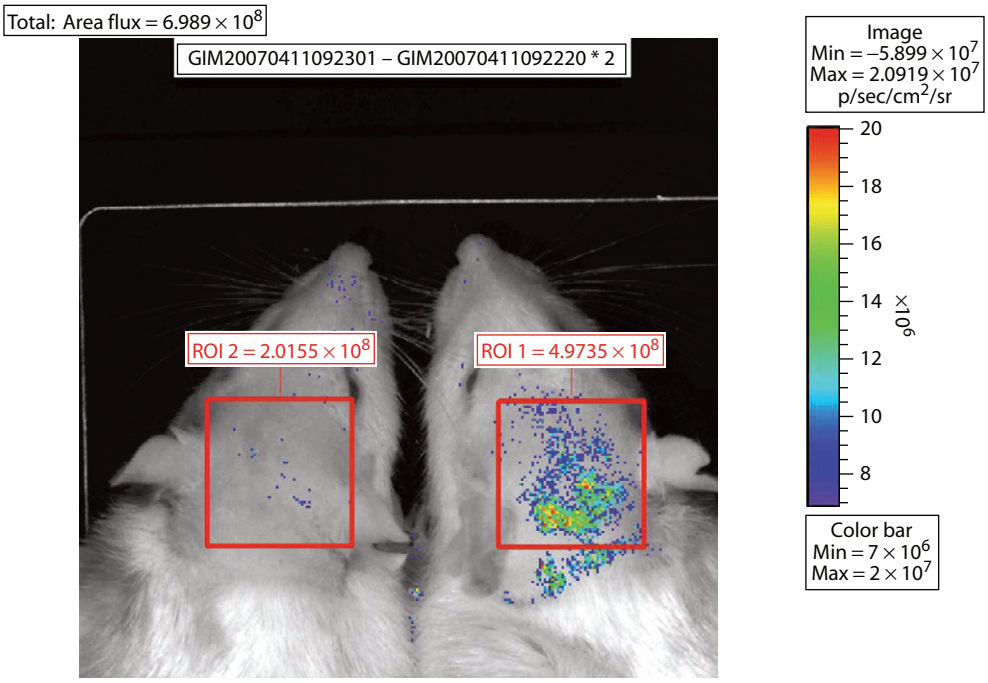

Figure 4 Evaluation of the fluorescence intensity of grafted pES/GFP$^+$ cells. Images were monitored with the continued tracking of the same rats with In Vivo Imaging System (IVIS) 50 for 3 months. (Left: control; Right: treatment). ROI: region of interest.

Table 1 Summary of the neural differentiation protocol, transplantation results, and behavioral recovery of ESC-derived DA neurons in animal models of PD[45,46,48,61,62,65,67,72,79–92]

ESC sources	Differentiation protocol	DA markers	Efficiency	PD animal model	Transplantation duration and results	Behavioral test	Behavioral recovery	Reference
Mouse ESC (CCE and EB5)	PA6-SDIA	TH, Nurr1, Pitx3, DA release	30 TH^+	6-OHDA lesioned striatum of mouse model	Integration with strong TH expression in PD mouse model	–	–	61
Mouse ESC (R1) transfected with Nurr1	5-stage method with SHH, FGF8, overexpression of Nurr1	TH, En1, AADC, DAT, Pitx3	78.2% TH^+	6-OHDA lesioned striatum of rat model	9 wk The DA neurons show electrophysiological and behavioral properties expected of neurons from the midbrain	Rotation, adjusting, paw-reaching, and cylinder test	Grafted with Nurr1 ES cells showed a significant improvement in the rotation, adjusting step, cylinder and paw-reaching tests	45
Mouse ESC (D3)	Small doses of undifferentiated cells	TH, NeuN, DAT, AACD, ALDH3	The no. of TH^+ was 2059 ± 626	6-OHDA lesioned MFB of rat model	9 wk ES-derived DA neurons caused gradual and developed teratoma-like structure	Rotation	Significant decrease in absolute numbers of amphetamine induced turning	79
Mouse ESC or nuclear transfer	Co-culture with BM-derived stromal, SHH, FGF8, bFGF, AA, BDNF	TH, DAT, AADC	No. of TH^+ was 23,000	6-OHDA lesioned striatum of rat model	8 wk Fiber outgrowth and neural subtype composition was detected	Rotation	More than 70% reduction in both amphetamine and apomorphine induced rotation	80
Mouse ESC (G4-2)	5-stage method with ITSFn, SHH, FGF8, AA	TH	32.5% TH^+	6-OHDA lesioned striatum of mouse model	12 wk Received a small number of grafted cells with tumor formation	Rotation	Significantly decrease in contralateral rotation	81
Mouse ESC (R1), transfected with Bcl-XL	5-stage method with ITSFn	TH, DA release	30.9% TH^+	6-OHDA lesioned SN and MFB of rat model	6 wk Bcl-ES-derived DA neurons exhibited more extensive fiber outgrowth	Rotation, stepping test	Reversal of behavioral symptoms than wild-type ES-derived DA neurons	46
Mouse ESC (EB3)	PA6-SDIA	TH	32% TH^+	6-OHDA lesioned SN and MFB of rat model	4 wk Grafted cells survived and innervated the host striatum	Rotation	Partial recovery from parkinsonian behavioral defects	87

(Contd)

Table 1 *(Continued)*

ESC sources	Differentiation protocol	DA markers	Efficiency	PD animal model	Transplantation duration and results	Behavioral test	Behavioral recovery	Reference
Human ESC (BG01)	PA6-SDIA	TH, DAT, Nurr1, Lmx1b, Pitx3, VMAT, AADC, DA release	87% TH^+	6-OHDA lesioned MFB of rat model	5 wk Small numbers of TH^+ cells survived, but larger numbers of cells for smooth muscle actin positive	Rotation	Failure of results in improvement of rotational behavior deficits	88
Human ESC (HSF-6)	PA6-SDIA, SHH, FGF8	TH, En1, Nurr1, Lmx1b, DA release and uptake	41% TH^+	6-OHDA lesioned SN and MFB of rat model	6 wk Grafted brains revealed that abundant hES-derived cells survived in the grafts, but none of them were TH^+	Rotation, step-adjustment	Failure of resulting in improvement of behavioral deficits	62
Primate parthenogenetic ESC (Cyno-1)	Co-culture with MS5, or MS5 overexpression of Wnt1, SHH, FGF	TH, En1, VMAT2	70% TH^+	Cynomolgus monkey injected with MPTP	7 mo Survival of cells both in PD and primate model	–	–	89
Monkey ESC	PA6-SDIA, FGF20, FGF2	TH, Nurr1, Pitx3, Lmx1b, DA release	24% TH^+	Cynomolgus monkey injected with MPTP	14 wk Survival of ES-derived DA neurons in the striatum	Neurological score	Significant functional improvement in PD primate model	82
Mouse ESC (MPI I)	PA6-SDIA	TH	The number of TH^+ was 330 ± 73	6-OHDA lesioned MFB of rat model	5 wk Graft recipients had tumors	–	–	90
Human ESC (SA002.5)	PA6-SDIA	TH, DA secretion, action potential	38% TH^+	6-OHDA lesioned striatum of rat model	13 wk Drafted with D16 developed teratomas, grafted with D20~23 remained healthy	Rotation	Reversal of lesion-induced motor deficits was not observed	67
Mouse ESC (D3, and Nurr1-expressing N2 or N5)	PA6-SDIA, overexpression of Nurr1, SHH. FGF, AA	TH, DAT, AADC, GIRK2, DA release, action potential	90% TH^+	–	4 wk Integration and generation of TH^+ cells after transplantation into striatum of mice	–	–	91

Mouse ESC (MM13 or DY-1) Human ESC (H9)	SDIA and transduction with Nurr1 and Pitx3	TH, DAT, VMAT2, DDC, DA release, action potential	15% ~ 45% DAT^+	6-OHDA lesioned unilateral intrastriatal mouse model	6 wk Low TH^+ cell recovery *in vivo*	Turning behavior	Significant behavioral recovery in PD mouse model	83
Primate ESC	Co-culture with mouse sertoli cells	TH, AADC, DAT, Nurr1, Lmx1b, DA release	90% TH^+	6-OHDA lesioned striatum of mouse model	2 mo Primate-derived DA neurons integrated into the mouse striatum, and survival of TH^+ cells in PD mouse model	–	–	92
Mouse ESC (R1)	5-stage method with ITSFn, SHH, FGF8	Lmx1b, TH, Pitx3, DAT	42% TH^+	6-OHDA lesioned striatum of rat model	32 wk Grafted TH^+ cells with elaborate dendritic processes and midbrain phenotype	Rotation	Sustained behavioral effects at 12 weeks after grafting and this change was stable for 32 weeks	84
Human ESC (H9)	FGF8, SHH, Wnt3a, AA, cAMP, TGFβ3, BDNF, GDNF	TH Lmx1b En1 ALDH2 Nurr1 Ptx3 Foxg1	43.4% TH^+	6-OHDA lesioned MFB of rat model	5 mo DA neurons present in the graft exhibited a midbrain, or nigra, phenotype	Rotation, adjusting step test, cylinder test	Behavioral improvement was correlated with the DA neurons present in the graft	65
Monkey ESC (CMK6)	FGF2, EGF	TH, DAT AADC	70% TH^+	Cynomolgus monkey injected with MPTP	6 mo Grafted cells differentiated into TH^+ cells and axonal outgrowth in the brain	Primate Parkinsonism rating scale	Modest behavioral improvement at 12 weeks after transplantation	72
Murine ESC (CCE)	RA induced	TH	–	6-OHDA lesioned MFB of rat model	8 wk TH^+ in engrafted cells after transplantation, and ultrastructural examination confirmed that the cells gained neuronal and glial appearance	Rotation	Parkinsonian rats had a significant decreased behavioural tests when subjected to the transplantation with retinoic acid-treated ES cells	85
Sox 1-GFP knock-in mice ESC (46C)	SHH and FGF 8b in SFEB	TH, DAT	18.7% TH^+	6-OHDA lesioned MFB of rat model	8 wk TH^+ neurons were found within the grafted site and all TH^+ profiles coexpressed the mouse-specific antibody	Rotation	A significant decrease in absolute numbers of drug-induced turning was seen in the transplantation group compared with control animals	86

(Contd)

Table 1 *(Continued)*

ESC sources	Differentiation protocol	DA markers	Efficiency	PD animal model	Transplantation duration and results	Behavioral test	Behavioral recovery	Reference
Pig ESC (M215-3 and GFP$^+$ 10)	Directed differentiation with RA, SHH, FGF	TH, DA	89.4% TH$^+$	6-OHDA lesioned MFB of rat model	3 wk Dopaminergic differentiation of grafted pES/GFP$^+$-derived cells was observed as determined by anti-TH and anti-DA	Rotation	Grafted with the pES/GFP$^+$-derived mature neurons showed a stably decrease relative rotation and resulted in a functional recovery from Parkinsonian behavioral defects	48

6-OHDA: 6-hydroxy dopamine; AA: ascorbic acid; AADC: aromatic amino acid decarboxylase, mature dopaminergic neuronal phenotype marker; ALDH2: aldehyde dehydrogenase, an enzyme expressed by midbrain dopaminergic progenitors; BDNF: brain-derived neurotrophic factor; BM: bone marrow; DA: dopamine; DAT: dopamine transporter, mature dopaminergic neuronal phenotype marker; DDC: dopa decarboxylase, midbrain DA marker; EGF: epidermal growth factor; En1: engrailed-1, mid/hindbrain transcription factor; FGF and bFGF: basic fibroblast growth factor; Foxg1: forebrain transcription factor; GDNF: glial cell-derived neurotrophic factor; ITSFn: insulin-transferrin-selenium fibronectin; Lmx1b: transcription factor regulating the development of midbrain DA neurons at early developmental stage; MFB: medial forebrain bundle; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NE: neuroepithelial cell; NeuN: neuronal nuclei marker; Nurr1: nur-related factor 1, transcription factors expressed in midbrain DA neurons; PA6: mouse stromal cell line; Pitx3: transcription factors expressed in midbrain DA neurons; SDIA: stromal cell-derived inducing activity; SFEB: serum-free embryoid body; SHH: sonic hedgehog; SN: substantia nigra; TGF: transforming growth factor; TH: tyrosine hydroxylase, mature dopaminergic neuronal phenotype marker; VMAT2: vesicular monoamine transporter, mature dopaminergic neuronal phenotype marker.

behavioral defects through the grafted mouse or monkey ES-derived cells.[72,87] Some studies even show failure in improving rotational behavior deficits when animals were transplanted with human ES-derived dopaminergic cells.[62,67,88] These inconsistent results imply the cell purification, cell population, induction protocol, survival and functional exhibition of grafted DA neurons are crucial for PD therapy.[48] The ES cell transplantation in the animal model of PD proves that this method is capable of relieving symptoms and offers real approach for cell replacement in regenerative diseases in the future.

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