Advances in stem-cell–generated transplantation therapy for Parkinson’s disease

Maria Sundberg & Ole Isacson†
Neuroregeneration Research Institute, McLean Hospital/Harvard Medical School, Belmont, MA, USA

Introduction: Human pluripotent stem cells have the potential to differentiate into different cell lineages of the human body, including dopaminergic (DA) neurons. Previous studies have shown that stem-cell–derived DA neurons can improve the motor deficits of Parkinson’s disease (PD) animal models. That is why current research interests focus on the development of stem-cell–derived neural cells for transplantation therapies for PD patients.

Areas covered: This review article emphasizes the safety and efficacy requirements of human pluripotent stem-cell–derived neural cells and usage of reliable preclinical animal models prior to clinical trials. The current advances and hurdles related to cell production, differentiation and transplantation are also summarized.

Expert opinion: Before entering the clinic, transplantable cell populations must be differentiated and characterized according to good manufacturing practice (GMP) regulations both in vitro and in vivo. Taking into account the rapid development of the stem-cell field and technological improvements in cell preparations and GMP facilities, we think that pluripotent stem-cell–derived DA neurons will offer a relevant cell therapy option for treatment of PD in the near future.

Keywords: cell banking, cell sorting, dopaminergic neurons, good manufacturing practices, human-induced pluripotent stem cells, neural differentiation, Parkinson’s disease, pluripotent stem cells, transplantation, xeno-free differentiation


1. Introduction

This review article highlights the recent progress of stem-cell therapy development for Parkinson’s disease (PD). PD is a movement-related disorder that results primarily from the death of dopaminergic (DA) neurons in the substantia nigra (SNc), a region in the ventral midbrain (VM). During the disease process, other parts of the brain are also affected, causing degeneration of non-DA neurons in the brainstem and cortex; and the peripheral nervous system is also involved, for example, in the gut and heart. Usually, the motor symptoms appear after decades of slow degeneration of the DA neurons and their connections. Current treatment options for PD focus on controlling motor symptoms with the dopamine precursor Levodopa, dopamine agonists or surgery (deep brain stimulation). There is currently no restorative treatment for PD, which is why current research efforts are focused on the development of cell transplantation therapies. Human fetal VM-derived neural cells and human pluripotent stem cells have been shown to be efficient for derivation of specific neural cell populations both in vitro and in vivo. In the following sections, important steps to assure the safety and efficiency of stem-cell–derived cell products for clinical use will be discussed: i) selecting an optimal cell source; ii) development of efficient differentiation protocols for producing functional DA neuron cell
populations; iii) optimization of purification protocols to eliminate harmful cells from the grafts; iv) use of reliable PD animal models; v) optimization of the protocols for clinical use according to established regulations for stem-cell–derived cell products; vi) stem-cell banking; and vii) good manufacturing practices (GMPs).

2. Parkinson’s disease

PD is often defined as a parkinsonian syndrome that is idiopathic, although some cases have a genetic origin such as, for example, mutations in LRRK2, PINK1, Parkin, SNCA, MAPT and HLA-DR [1]. The most common symptoms of PD are tremor, rigidity, slowness of movement, difficulty walking and uneven gait [2,3]. Sleep problems, depression and dementia are also common for PD patients [4,5]. In general, the human CNS is particularly vulnerable to injuries and degenerative processes. Also, the ability of the adult brain in primates and humans for endogenous cell replacement is very limited, and exclusively occurs in the hippocampus [6-9]. However, the brain’s capacity for remodeling the signaling networks and forming new synaptic connections between neurons makes the brain an attractive target for cell transplantation therapies [10-15].

3. Finding an optimal stem-cell source

Several different sources of stem cells have been used for DA neuron derivation and differentiation for the development of cell therapy for PD. The most commonly studied human cell sources for DA neuron derivation are human fetal VM cells, human embryonic stem cells (hESCs) and human-induced pluripotent stem cells (hiPSCs) [11-13,16-18].

3.1 Human fetal ventral midbrain cells

Human fetal VM cells can be isolated from the developing brain tissue of aborted fetuses after receiving donor consent from the women undergoing surgical termination of pregnancy, between 6 and 10 weeks of gestation. Previous transplantation studies have shown that human fetal VM tissue pieces and cell clusters can be transplanted to the PD patient brain. In addition, some studies have shown that enzymatic or mechanical dissociation of fetal VM tissue pieces to single-cell suspensions improve the cell survival, vascularization and integration of the cells to the brain after transplantation [19]. Previous clinical trials with fetal VM DA neurons have shown that these grafts can produce high levels of dopamine, and their survival and ongoing functional effects have been demonstrated for over 14 years in patients’ brains [20,21]. Some studies have also described significant clinical improvements in motor symptoms [13,20,22]. However, some reports show that a few patients have developed abnormal involuntary movements (gait-induced dyskinesia [GID]) [23], as well as poor improvements for behavioral deficits [24,25].

Moreover, human fetal studies possess significant ethical and religious concerns related to abortion regulation and destruction of embryos. That is why this review article concentrates on describing differentiation protocols and cell-sorting strategies for an alternative cell source, human pluripotent stem cells, which has great potential for future cell transplantation therapies.

3.2 Human embryonic stem cells

The first hESC lines were derived successfully in 1998 by James Thomson’s laboratory [26]. hESCs are derived from the inner cell mass of the fertilized oocyte. hESCs are pluripotent cells, which means that these cells can differentiate toward all three different germ-layer cell types of the human body: ectoderm-, mesoderm- and endoderm-derived cells, excluding trophoblasts [27]. Stem cells have been derived and cultured on top of feeder cells or different protein matrices, like matrigel and vitronectin, which support cells’ maintenance in the undifferentiated stage [26,28]. hESCs are commonly characterized by their gene expression of OCT4, SOX2, NANOG, GDF3 and DNMT3b and protein expression of SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81. hESCs have high telomerase activity levels and they have been shown to retain normal karyotype in prolonged culturing in vitro [29].

Due to the stem cells’ efficient self-renewal ability they can be further passaged and cultured in the undifferentiated stage in vitro for prolonged periods of time [27]. In vivo pluripotent hESCs form teratomas, which contain ectoderm, endoderm and mesoderm cell types [26]. Moreover, in vitro hESCs form embryoid bodies that consist of all three human germ-layer cell types. Thus, hESCs can be envisioned as a cell source for differentiation of different cell types in vitro, including derivation
of neural cells and specialized DA neurons for transplantation purposes [11,12,18,30].

3.3 Human-induced pluripotent stem cells
The first hiPSC lines were derived in 2007 by Dr. Yamanaka’s laboratory [31]. These cell lines were derived from human fibroblasts using pluripotency-inducing transcription factors OCT4, c-MYC, SOX2 and KLF4 attached to retrovirus vectors that were used to infect the fibroblasts. Other combinations of transcription factors have also been used to induce pluripotency, such as the combination of OCT4, SOX2, NANO2 and LIN28 [32]. iPSCs resemble hESCs in their morphology, and gene and protein expressions, proliferation capacity and telomerase activity [33]. The pluripotency of iPSCs was confirmed by teratoma formation capacity and differentiation potential to produce cell types from ectodermal, endodermal and mesodermal lineages. After establishing the first hiPSC lines, the gene pattern to induce pluripotent stem cells has been modified to contain only genes for Oct4, Sox2 and Klf4, since c-MYC is a powerful oncogene that increases the tumorigenicity of the cells [33]. Also, for the induction of the cells’ pluripotency and increasing safety, previously used retrovirus vectors have been replaced with non-integrating sendai-virus vectors [34], adenovirus vectors [35], plasmids [36], transposons [37], episomal vectors [38], chemical compounds [39] and recombinant proteins [40] to produce mutagenesis and virus-free iPSCs. These reprogramming technologies make it possible to isolate somatic cells from patients suffering from severe diseases and produce patient-specific iPSC lines, for example, from patients with PD [41-44]. The PD patient-specific iPSCs that carry mutations for PARK2, PINK1, LRRK2 and SNCA have been studied intensively for assessment of neural cell vulnerability for oxidative stress and mitochondrial dysfunction and cell survival after treatment with different drugs and toxins in vitro [42,45]. Importantly, human iPSC-derived DA neurons have also been shown to be functional in vivo and these cells improve the motor functions of PD animals after transplantation [17].

4. Dopaminergic neuron derivation

4.1 Differentiation and characterization of pluripotent stem-cell-derived DA neurons
For generation of human pluripotent stem-cell-derived DA neurons, recently published protocols are mimicking embryonic development in a dish by activating transcription factor pathways important for VM DA neuron derivation [11,12,16,30]. Figure 1 provides a schematic presentation of the pathways and markers important for VM DA neuron derivation from pluripotent stem cells.

The floor plate (FP) selectively exhibits neurogenic potential, and it has been shown to be a source for VM DA neuron derivation [46,47]. During FP development, cells have a unique, flat morphology and express FP-specific markers, including sonic hedgehog (SHH), Foxa2, F-Spondin and Ntrin-1 [46,47]. FP induction of pluripotent stem cells requires highly activated SHH (Shh C25 II/SHH C24 II) exposure that induces FOXA1/2 expression in the differentiating cells [47]. In our previous study, we have also shown that FP induction is sufficient with the small-molecule SAG, which is a chlorobenzothiophene-containing Hh pathway agonist [18]. Derivation of VM DA neurons from FP cells also requires effective neurogenic induction. Neural induction of hESCs in the feeder-free cultures has been shown to be effective with dual SMAD (homolog to Caenorhabditis elegans protein SMA (from gene sma for small body size) and homolog to Drosophila protein; mothers against decapentaplegic [MAD]) inhibition with the recombinant protein noggin and small molecule SB431542 [11,48]. Noggin is a specific inhibitor for bone morphogenetic protein (BMP) receptor and SB431542 inhibits transforming growth factor β (TGFβ) signal transduction. Both of these molecules have a downstream effect in inhibiting the SMAD signaling pathway. SMADs are intracellular proteins that transduce extracellular signals from ligands to the nucleus where they activate downstream gene transcription. Recombinant protein noggin can also be replaced with a small-molecule LDN-193189 [11,18]. LDN-193189 is a structural analog of Dorosomorphin 4, and it inhibits BMP-induced SMAD-, p38- and Akt signaling.

During VM DA neuron development it has been shown that wnt-signaling is important for DA neuron differentiation [49]. In addition, it is important to have the anterior-posterior axis defined in terms of retinoic acid concentration, and the midbrain regionalization appropriate according to FGF8 a/b specification [16]. In the presence of wnt-signaling, glycogen synthase kinase 3 (GSK-3) is inhibited, and unphosphorylated-catenin enters into the nucleus activating LEF/TCF transcription factors. GSK-3 inhibition in VM precursor cultures has been shown to increase differentiation of DA neurons [49]. Recent studies have shown that a small molecule, CHIR99021, which specifically inhibits GSK-3, can be used for activation of the wnt-signaling pathway in hESC-derived FP-derived neural cell cultures [11,12,18]. Expression of anterior-posterior patterning factor OTX2 has been shown to be upregulated during this differentiation process [18]. Previous studies have shown that Otx2 is required for derivation of VM DA neuron precursors from FP origin [46]. The activity of Otx2 to induce neurogenesis in FP is co-regulated by induction of Lmx1a, which is a transcription factor required for proneural gene expression of FP cells [46]. Lmx1a regulates DA neurogenesis by inducing expression of proneural factors Math1 and Acdn2 [46]. Currently established DA neuron differentiation protocols for hESCs shows that, in the presence of CHIR99021, the expressions of OTX2, FOXA1, FOXA2, LMX1A, MSX1 and NGN2 are upregulated in the FP-derived neural precursor cell cultures, as well as co-expression of LMX1A/FOXA2 and OTX2/FOX2A [11,12,18,30].
During VM development, Foxa1/2 expression remains present in the postmitotic VM DA neurons and it regulates the differentiation and phenotypic maturation of VM DA neurons by controlling the expressions of Nurr1, En1, tyrosine hydroxylase (TH) and aromatic amino acid decarboxylase (AADC) [50,51]. During embryonic brain development, Nurr1 is expressed in the ventral midline of E10.5 mice and also in the forebrain and hindbrain of developing mice embryos. Specifically, Nurr1 is expressed in A8-A10-type DA neurons and its expression is crucial for normal development of ventral DA neurons [52]. Also, during embryogenesis, EN1 and EN2 have been detected in the ventral midline where the VM DA neurons arise [12]. Wnt-signaling pathway activation and correct timing and concentration of GSK-3 inhibitor are also important for the induction of NURR1 and EN1 expression in human FP-derived FOXA2/LMX1A+ cells [11,12,18,30]. Differentiated DA neurons express TH, which is the enzyme that catalyzes the initial rate-limiting step in the biosynthesis of catecholamines in the brain, calbindin is a calcium-binding protein expressed in the vast majority of TH+ A10-type DA neuron subtypes [54,55]. GIRK2 has been reported to be expressed in TH+ A9-type DA neurons of the SNc [55]. A9- and A10-type DA neurons can be distinguished by their morphology, localization and GIRK2 or calbindin expression [54,55]. The functional improvement and innervation of the striatum is mainly caused by A9-type nigral DA neurons, whereas the A10-type VTA cells project to extrastriatal forebrain areas [54,55]. It has been shown that the established differentiation protocols for pluripotent stem-cell-derived FP VM DA neurons produce cells that mature in vivo into TH/GIRK2+ VM DA neurons and innervate the host striatum with TH+ fibers [11,12,18]. Although these methods produce functional VM DA neurons quite efficiently, the heterogeneous cell populations possess risk for tumor formation or other graft-induced side effects after transplantation [18,56]. Thus, efficient sorting methods should be included in the protocol to assure the safety and purity prior to clinical translation.

5. Safety issues of stem-cell-derived DA neuron transplantations

5.1 Tumor formation
Pluripotent stem-cell-derived cell populations possess risks for causing tumors after transplantation, since the cell
populations can contain undifferentiated cells or proliferating non-neural cells [57,58]. Especially, Oct-4, SSEA-4 and Tra-1-60-positive cells have been shown to cause teratomas in vivo [58]. Furthermore, it has been shown that inefficient neural differentiation protocols may result in the appearance of two morphologically distinct populations that are either pluripotent stem-cell-derived DA neuron cell populations prior to transplantation enables the elimination of serotonergic neurons and increases the safety of the grafts [18].

6. Purification of pluripotent stem-cell-derived neural cell populations with cell sorting

6.1 Green fluorescent protein-tagged cell sorting
As our and other groups’ studies have shown, pluripotent stem-cell-derived neural cells entail risks for tumor formation upon transplantation if the cell population contains undifferentiated cells or proliferative non-neural cells [57,58]. To solve this issue, several sorting methods have been developed for enrichment of differentiated neural cell populations using FACS or MACS. For example, different transgenic ESC lines with green fluorescent protein (GFP)-tagged promoters have been produced for enrichment of DA neurons: Hes::GFP, Nurr1::GFP, Pitx3::GFP [56,64]. According to these studies, the Nurr1::GFP sorting resulted in neuronal cell grafts that had the greatest amount of DA neuron survival and robustly induced recovery of motor deficits in hemiparkinsonian mice. Hex::GFP sorting resulted in more immature cell type and Pitx3::GFP sorting resulted in more mature cell population that did not survive as well as Nurr1::GFP cells after transplantation. Nurr1::GFP sorting also induced expression of known midbrain DA neuron genes and established novel marker for VM precursor cells named UNSEX4.1 [56]. Although this sorting strategy is efficient for enrichment of functional DA neuron precursors for transplantation, genetic manipulation of cells generates safety risks, like increasing risk of chromosomal mutations and tumorigenicity of cells. That is why surface protein-based sorting is considered to be more optimal for future clinical applications, and screening of suitable markers for specific subpopulations is important [59,60].

6.2 Surface antigen-based cell sorting
Corin is an early VM DA progenitor marker that can be used to enrich DA progenitors with FACS. Corin-positive VM DA neurons originate from mesencephalic ventral midline that have FP characteristics and co-express Lmx1a and FP marker FP4 [46]. A previous study has also shown that sorted corin+ ESC-derived neural progenitors are proliferative in vitro and able to differentiate into TH-positive neurons that also express the VM DA neuron markers Pitx3 and DAT [46]. However, the expression of corin is limited to a short period of time during ESC-derived DA neuron development and its expression is very low [46]. This limits the efficiency of corin+ sorting and reduces the scalability of the method for clinical use.

During neuronal differentiation of hESCs, the expressions of pluripotency markers Tra 1-60, Tra-1-81, SSEA-1,
SSEA-4/3 and CD326 (EpCam) are downregulated [60,61]. After neural induction, the neural stem cells (NSCs) express CD184, and after further differentiation the cells express neuronal markers like CD56 and CD271 [60]. CD184 is a marker also known as the chemokine receptor CXCR4, and it is characterized as a receptor protein modulating cell growth and migration of neural cells in the CNS [65]. CD271 is a marker for nerve growth factor receptor, a protein that has been found localized to neuronal axons, Schwann cells and peripheral nerves. CD56 is a marker for neural cell adhesion molecule (NCAM), which is a homophilic binding glycoprotein expressed on the surface of hESC-derived NSCs and neurons [60,61,66]. NCAM has a role in cell–cell adhesion, neurite outgrowth and synaptic plasticity. Previous studies have also shown that NCAM+ sorting enriched hiPSC-derived TH+ DA neurons and these cells survived after transplantation into 6-hydroxydopamine (6-OHDA) rat brain and improved the motor symptoms of the animals [17].

Considering the fact that there exist over 200 different CD markers, several combinations of markers have been described for isolating differentiating neural cells derived from human pluripotent stem cells. For example, sorting with a combination of CD184+/CD271+/CD44+/CD29+, the NSCs could be isolated from hESC- and hiPSC-derived neural cell populations [67]. Also, neurons can be sorted with a CD184+/CD44+/CD15low/CD29+ selection, and glial cells can be sorted with a CD184+/CD44+ selection [67]. CD44 is a marker for a receptor for hyaluronic acid. The protein encoded by this gene is a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration, and this marker has been localized in glial precursor cells derived from hESCs [68,69]. In addition to this, another combinatorial sorting strategy has been established to distinguish subpopulations of NSCs, neural crest cells and neurons from heterogeneous hESC-derived cell populations [59]. Here the hESC-derived NSCs were sorted with a CD15+/CD24low/CD29high selection, hESC-derived neural crest cell populations were sorted with a CD15+/CD24low/CD29high selection and neuronal cells were isolated with a CD15+/CD24low/CD29high selection and neuronal cells were isolated with a CD15+/CD24high/CD29low selection [59]. CD15 is also known as SSEA-1, which is a carbohydrate adhesion molecule that is expressed on glycoproteins, glycolipids and proteoglycans. CD24 is a marker for heat-stable antigen, which is a cell adhesion molecule, and not specific for neurons alone [60]. CD29 is a marker for β-integrin and it is expressed in a wide variety of cell types: epithelial cells, lymphocytes, smooth muscle cells and FP cells. After transplantation into rodent striatum, the hESC-derived NSCs, CD15+/CD24low/CD29high, formed tumors in the brain while sorted CD15+/CD24high/CD29low neurons did not form any tumors and integrated into host brain tissue by extending neuronal processes [59]. These results suggest that when sorted with a combination of CD markers, the hESC-derived neuronal cells possess a reduced risk for tumor formation.

In a recent study, we have demonstrated that hESC- and hiPSC FP-derived neural cell populations contained subpopulations of NCAMlow, CD29+, NCAM+ and NCAM+/CD29low cells [18]. From these subpopulations, the NCAMlow population contained cells that expressed KDR, a marker for mesodermal cells. Also, a low level of pluripotency marker SSEA-4 was detected in the NCAMlow subpopulation. The NCAM+ population included NSCs/precursors that were positive for CD184+/CD133low [18,60,67], and also neuronal cells that were CD24+/CD29low [18,59]. Gene expression profiling of these subpopulations showed that NCAM+/CD29low cells had significantly higher expression levels of DA neuron-specific mRNAs: Nurr1, Girk2, Pitx3 and Th, compared to an unsorted neural cell population, NCAMlow, or CD29+ cell populations [18]. Sorting with NCAM+/CD29low significantly enriched the number of FOXA2/Th+ and En1/Th+ DA neurons compared to an unsorted cell population (10 – 20% positive cells before sorting vs 40 – 50% positive cells after NCAM+/CD29low sorting) [18]. Importantly, our study showed that NCAM+/CD29low selection reduced the number of calbindin-positive A10-type DA neurons and reduced number of 5-HT-positive serotonergic neurons in hiPSC-derived DA neuron populations compared to unsorted cell populations [18]. In addition, prior to transplantation, NCAM+/CD29low sorting eliminated non-neural tumorigenic cells from the grafts and increased significantly the number of TH+ cells, and these cells improved behavioral deficits of the 6-OHDA rats [18]. As described above, none of the selected CD markers are specific for cell type by themselves (see Table 1 for example). It will be important to use combinations of at least two different markers to assure efficient purification and enrichment of DA neurons derived from pluripotent stem cells.

7. PD animal models for studying the safety and efficiency of stem-cell-derived DA neurons

7.1 6-OHDA rodents

The unilateral injection of a dopamine-specific neurotoxin 6-OHDA into the medial forebrain bundle degenerates DA neurons in the rodent striatum. Local injection is required because 6-OHDA does not cross the blood–brain barrier. Bilateral injections result in high mortality rate of the animals; thus, the unilateral injection of 6-OHDA is a common practice to produce the lesion. 6-OHDA has oxidative properties, and after entrance to the neuron 6-OHDA accumulates in the cytosol and undergoes prompt oxidation, promoting a high rate of hydrogen peroxide formation that is highly toxic for the cells. Due to the contralateral projections, a right-sided lesion causes motor function deficit on the left side, which can be assessed by using different behavioral assays. The apomorphine- and amphetamine-induced rotation tests provide readouts for the severity of the lesion, as well as of the functionality of transplanted DA neurons. The systemic administration of apomorphine leads to a biased circular movement toward the side contralateral to the lesion through dopamine agonism
(contralateral rotations). In contrast, amphetamine-induced rotational assays can be used to monitor dopamine release from neuronal storage. After amphetamine injection, a lesioned rat will rotate toward the lesioned side (ipsilaterally), due to a predominant contralateral dopamine release from the intact SNc. Several studies have shown that grafting of functional pluripotent stem-cell–derived DA neurons ipsilaterally into the lesioned brain restored the dopamine input to the striatum and reduced the number of ipsilateral rotations [11,12,17,18]. In addition, a highly functional ipsilateral graft in a lesioned animal may even lead to contralateral amphetamine-induced rotations toward the unlesioned side, due to excessive dopamine output by the graft [11,12,18]. More accurate information can be gained with non–drug-induced spontaneous movement tests that can be used to evaluate complex motor behavior by determining parameters such as forelimb usage, side bias and appropriate adjustment steps. Although DA depletion in 6-OHDA rats causes significant reduction of contralateral limb use, several studies have shown that pluripotent stem-cell–derived DA neuron grafts improve these behavioral deficits 16 – 20 weeks after transplantation [11,12,18].

Compared to mice, there are several reasons why rats are preferred for studying cell survival, safety and efficacy after transplantation in a PD model. i) Compared to mice, the neuronal circuitry of rats more closely resembles that of humans; and ii) rats are less prone to anxiety and become easily familiar with handling, which represents a major advantage for behavioral assessments. Also, for evaluation of the safety of the cell grafts, it is important to select reliable animal models. Previous studies have shown that when using inefficient neural differentiation protocols for pluripotent stem cells, the heterogeneous cell grafts developed teratomas in the 6-OHDA rat brain [57]. Especially when using immunocompromised mice, the propensity for tumor formation tends to increase if any pluripotent stem cells are present in the graft [56]. That is why

<table>
<thead>
<tr>
<th>Antigen</th>
<th>hESC/hiPSC</th>
<th>NSC/NPC</th>
<th>Neuron</th>
<th>Glial cells</th>
<th>MSC</th>
<th>HSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>x</td>
<td>x,¤</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD9</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD10</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD13</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD15</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD24</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD29</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD31</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD38</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD49b</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD49d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD49f</td>
<td>x</td>
<td>x,¤</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD56</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD59</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD61</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD71</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD90</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD105</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD106</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD117</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD133</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD135</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD140a</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD144</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD146</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD166</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD184</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD200</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD271</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD326</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

See refs [54,55].

hESC: Human embryonic stem cells; hiPSC: Human-induced pluripotent stem cells; HSC: Hematopoietic stem cells; MSC: Mesenchymal stem cells; NPC: Neural precursor cell; NSC: Neural stem cells; X: Human cells; ¤: Animal cells.
prolonged safety studies are required to assure that the produced pluripotent stem-cell-derived DA neuron cell grafts are safe for clinical translation. However, rodent studies are limited to 6 months, whereas nonhuman primates can be studied for > 3 years after cell transplantation. Also, more complex behavioral assays can be performed for nonhuman primates, for example, paw reaching tests and more advanced cognitive tasks relevant for evaluating motor scores in parkinsonism. Thus, when performing preclinical animal studies for the safety and efficiency of pluripotent stem-cell-derived DA neuron grafts, the tests should be performed in a reliable host that offers readouts comparable to human conditions.

7.2 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine-nonhuman primates
1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a synthetic neurotoxin that induces parkinsonism in humans [70] and also in monkeys [71]. DA neurons have been shown to be especially sensitive to MPTP. As a result of MPTP administration, DA neurons die in the striatum. MPTP is transformed by monoamine oxidase B into its active metabolite, 1-methyl-4-phenylpyridinium ion (MPP+), after crossing the blood–brain barrier. Dopamine transporter carries MPP+ into DA neurons of the SNc pars compacta where it blocks mitochondrial complex I activity. Because of the neurochemical and behavioral parallels to human PD, the MPTP-lesioned nonhuman primate is considered the best model for PD [72] and is used to study the pathophysiology of PD and evaluate candidate treatments for PD. The primate species currently used in PD research include the *Macaca fascicularis* (cynomolgus), the *Chlorocebus sabaeus* (African green monkey also called the vervet monkey), the *Macaca mulatta* (rhesus), the *Saimiri sciureus* (squirrel monkey) and the *Callithrix jacchus* (marmoset).

To establish a nonhuman PD model, MPTP is administered at low doses several times per week for several months. MPTP administration will be continued until behavioral deficits appear and motor symptoms are quantified with parkinsonian rating scores. Animals are considered ‘cognitively impaired’ if they show at least a 15% decrease in cognitive sonian rating scores. Animals are considered ‘cognitively impaired’ if they show at least a 15% decrease in cognitive performance can be monitored with a computer-controlled touch-screen battery of tests that have been validated in this model. Locomotor activity can be measured with infrared monitoring, electromyographic analysis and analysis of traveled distances with video-recordings [72]. Also, several behavioral tests for analyzing motor functions as well as nonmotor functions have been developed, for example, food-retrieval tasks [75].

Functional brain imaging with positron emission tomography (PET) scanning with 18F-DOPA and 11C-DTBZ PETs provides valuable information of DA depletion in monkeys with MPTP-induced parkinsonism. Previous studies have shown that significant correlations exist between i) total parkinsonian symptom scores and mean striatal [18F]-F-DOPA uptake and between ii) lateralized limb movements and contralateral [18F]-F-DOPA uptake in MPTP-lesioned monkeys (for left striatum and right limbs) [76]. Also, it has been shown that 11C-DTBZ is a more stable and ‘structural’ marker of DA transmission as compared to 18F-DOPA [77]. Results have shown that the VMAT2 receptor 11C-DTBZ is affected in the course of the disease well before reduction in 18F-DOPA uptake is detected [78]. These PET scanning approaches have also been used to evaluate the effect of early cell-therapeutic intervention and putative neuroprotective effects of cell grafts in the striatum [58].

Studies in MPTP-lesioned monkey brain have shown that hESC- and hiPSC-derived DA neuron cell populations survive 1 month after transplantation and TH+ fibers extend to the host striatum from the graft site [11]. However, this study also reported Iba+ microglia infiltration to the grafts [11], suggesting immunosystem activation and initiation of immunorejection. In addition, another study has shown that after 3 months of hESC-derived neuronal cell transplantation into MPTP monkey brain, the xenografts of human stem-cell derivatives in CsA-suppressed rhesus monkey brain were mostly rejected [79]. These studies highlight the importance of proper immunosuppression paradigms and the fact that monkeys have complex immunological systems [79,80]. Related to these issues, development of autologous cell grafts can overcome the problems related to insufficient immunosuppression and improve the survival of the cell grafts. Thus, our group and others have shown that iPSCs can be derived from the skin fibroblasts of nonhuman primates, and these iPSCs can be differentiated into DA neurons [18,81,83]. After autologous transplantation these cells survive from 6 – 12 months [18,82] up to 24 months [83] in the striatum of PD monkeys, without any immunosuppression and without signs of active immune rejection. Importantly, 1 year after transplantation, neither tumor formation nor graft overgrowth was detected in the striatum [18]. Also, surviving FOXA2/TH+ DA neurons were detected in the graft with TH+ fiber outgrowth [18]. Taken together, the MPTP-treated nonhuman primate models of PD clearly represent valuable pathophysiological as well as behavioral models for PD and are important for preclinical safety and efficacy testing of both iPSCs and hESC-derived DA neurons prior to clinical translation.

8. Clinical requirements for pluripotent stem-cell-derived DA neuron cell products

8.1 Good manufacturing practices
For the future, clinical application human stem-cell-based products need to be developed according to GMPs [84]. This means that the whole manufacturing process for stem-cell-derived products will be performed under quality-controlled conditions in clean room facilities, using established validated protocols and proper documentation, and that the methods are reproducible and performed with defined and traceable raw materials and xeno-free products [84]. Also,
extensive quality control for each cell batch should be performed before releasing the products for clinical use to ensure i) absence of bacterial and viral infections and endotoxins; ii) expression of genes and proteins characteristic for the cells; iii) stability and viability of cells after freezing/thawing; and iv) in vivo assessments for safety and efficiency of the cells in animal models. Table 2 describes the current advances and hurdles related to pluripotent stem-cell–derived VM DA neuron translation to clinical use.

8.2 Establishment of MHC-matched pluripotent stem-cell banks
Previous organ transplantation studies have shown that transplantation of tissue from unrelated donors will activate the host’s innate and adaptive immunological responses, leading to inflammation, cell death and ultimately rejection of the transplanted tissue [85]. The key player in the immune system is the MHC region, which contains around 200 genes that are central to immune recognition. In humans, the human leukocyte antigen (HLA) system presents foreign proteins to antigen-presenting cells and T-lymphocytes, which activate immune responses. Similar to whole-organ transplantation, the clinical transplantation of hESCs- or iPSC-derived tissues that express foreign HLA may result in immunological rejection [86]. Although hESCs express low levels of HLAs, upon differentiation, the HLA expression is increased on the cell surfaces [87]. For the clinical translation of stem-cell–derived products, establishment of HLA-matched cell banks will facilitate the match between donors and recipients and reduce the likelihood of immune rejection after transplantation [86].

8.3 Future clinical translation of hiPSC-derived cell populations
Compared to hESCs, hiPSCs possess several advantages for the future development of cell transplantation therapies for neurodegenerative diseases. hiPSCs are derived from adult cells without the use of human fertilized eggs or embryos, which diminishes the ethical concerns related to destruction of embryos. The iPSC technologies also facilitate the development of autologous cell therapies for patients by using their own cells. However, currently, the time and money required for reprogramming, characterization and safety evaluation of individual iPSC clones limit their usage for autologous cell therapy. There is an almost unlimited cell supply to generate iPSC lines from skin biopsies, which offers the opportunity to produce MHC-matched iPSC lines for allogeneic cell graft production for clinical use similar to MHC-matched hESC lines [86,88]. There are also several cellular aspects that need to be studied before entering the clinic with hiPSC-derived cells. Among those are i) cells’ epigenetic memory after reprogramming [89]; ii) tumorigenicity of remaining pluripotent cells in the graft; iii) chromosomal mutations; and iv) insufficient reprogramming technology [90]. Insufficient reprogramming can induce abnormal gene expression in iPSC-derived cells and induce T-cell–dependent immune responses in syngeneic recipients [91]. In addition, in the case of derivation of hiPSC lines from PD patients carrying genetic mutations, it will be important to evaluate the safety of the differentiated DA neurons prior to transplantation. Related to this, the genetic modifications with zinc finger nucleases, TALENs and CRISP-Cas9 technologies will facilitate the correction of the mutations in iPSC lines, prior to differentiation to neurons [92]. However, there are safety risks related to chromosomal mutations and altered genotypes of modified cells. Thus, the genetic modification of the cells should be kept minimal prior to clinical translation. More research studies and technology development are also needed to increase the safety, identity and chromosomal stability of iPSC lines prior to clinical translation of these cell products. However, hiPSCs possess great potential for the future of clinical research, because of the opportunity to do autologous or closely matched cell transplantation in cell-organ systems of the human body.

8.4 Xeno-free culturing protocols
Previously, stem-cell lines were directly or indirectly exposed to animal substances during derivation or maintenance of cells in vitro. Some of these cell lines were cultured on top of mice fibroblast feeder cells and the colonies were passaged enzymatically [93]. Studies have shown that use of mouse fibroblasts may affect the differentiation capacity of cells and contaminate cell cultures with animal substances and increase the rejection prospects of these cells [94,95]. To overcome these problems, several research groups have used human foreskin fibroblasts for stem-cell derivation. Also, culture mediums have been supplemented with human serum or serum replacement to avoid the use of animal serum, and mechanical cell passaging has been used to avoid the use of enzymes [96]. Human fibroblasts secrete several factors to the cell cultures that affect cell maintenance in the undifferentiated stage and support colony formation of stem cells, such as TGF-3b and FGF2. However, currently developed feeder-free cultures for stem cells will make it easier to define the exact components and soluble factors affecting cells and help standardize the culturing conditions [97]. One of the most commonly used coating matrices for differentiation is the commercial xenogenic gel product Matrigel (BD Biosciences). Matrigel was originally derived from the mouse sarcomagelatinous protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells, and the exact composition of the factors included in this product has not been identified. To avoid the use of xenogenic Matrigel, several research groups have tested human extracellular matrix protein mixtures like vitronectin and laminin511 for stem-cell culturing and maintenance [98,99].

Although at the moment FDA regulations do not require culturing protocols to be totally xeno-free, the use of xenogenic undefined products for clinical-grade cell production is not recommended due to the existing risks of pathogen
Table 2. Clinical translation of human pluripotent stem-cell-derived DA neurons for treatment of PD patients.

<table>
<thead>
<tr>
<th>Cell manufacturing process</th>
<th>Achievements</th>
<th>Hurdles</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMP cell production</td>
<td>Establishment of clean room facilities, Derivation of feeder-free, xeno-free hESC-lines with defined culture conditions, HLA typing of hESC lines with PGD, Induction of pluripotency in somatic cells, Establishment of non-integrating viruses for transfection of the cells, Virus-free methods for delivering reprogramming factors, Zinc finger nuclease, TALENs, and CRISP – Cas9 technologies for correction of genetic mutations</td>
<td>High costs, Requires trained quality-control personnel and constant audits of facilities, SOPs and equipment, Establishment of HLA-matched cell-line banks requires characterization of large amount of cell lines, hiPSC line translation to clinics, Insufficient reprogramming methods, Risk of chromosomal mutations, Epigenetic memory, Risk of dedifferentiation</td>
</tr>
<tr>
<td></td>
<td>Establishment of efficient FP induction protocols for pluripotent stem cells, Establishment of dual SMAD-inhibition protocols to derive neural induction of pluripotent stem-cell-derived FP cells, Establishment of protocols to activate the wnt-canonical signaling pathway to derive VM DA neurons from FP cells, Development of xeno-free products, Knock-out serum replacement, Small molecules, B27 supplement, N2 supplement, Development of defined culturing medium; E8, mTESR, Development of suspension culturing protocols for differentiation of VM DA neurons, Cell sorting with FACS/MACS, Surface markers, Transfection of GFP-tagged transcription factors</td>
<td>The differentiation protocols contain undefined factors and xenogenic components, Matrigel, Recombinant proteins, Medium supplements, Derivation of a specific VM DA neuron subpopulation is not efficient enough, Currently existing protocols result in heterogeneous cell populations, Harmful cells after differentiation, Undifferentiated pluripotent cells, Non-neuronal cells, Serotonergic neurons, There exist significant line-specific differences in the efficiency of DA neuron differentiation, Lack of specific markers for VM DA neuron sorting, Challenges in optimization of the timing of cryopreservation and optimal time required to recovery after thawing/prior to transplantation of the cells</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Differentiation of DA neurons</th>
<th>Achievements</th>
<th>Hurdles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Select a GMP-level produced HLA-matched pluripotent stem-cell line</td>
<td>Establishment of efficient FP induction protocols for pluripotent stem cells, Establishment of dual SMAD-inhibition protocols to derive neural induction of pluripotent stem-cell-derived FP cells, Establishment of protocols to activate the wnt-canonical signaling pathway to derive VM DA neurons from FP cells, Development of xeno-free products, Knock-out serum replacement, Small molecules, B27 supplement, N2 supplement, Development of defined culturing medium; E8, mTESR, Development of suspension culturing protocols for differentiation of VM DA neurons, Cell sorting with FACS/MACS, Surface markers, Transfection of GFP-tagged transcription factors</td>
<td>The differentiation protocols contain undefined factors and xenogenic components, Matrigel, Recombinant proteins, Medium supplements, Derivation of a specific VM DA neuron subpopulation is not efficient enough, Currently existing protocols result in heterogeneous cell populations, Harmful cells after differentiation, Undifferentiated pluripotent cells, Non-neuronal cells, Serotonergic neurons, There exist significant line-specific differences in the efficiency of DA neuron differentiation, Lack of specific markers for VM DA neuron sorting, Challenges in optimization of the timing of cryopreservation and optimal time required to recovery after thawing/prior to transplantation of the cells</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Characterization of the cells</th>
<th>Achievements</th>
<th>Hurdles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Use of qualified characterization methods</td>
<td>Quality-controlled methods and high-throughput analyzing methods with established markers, qRT-PCR, Sequencing of the genome, Flow cytometry, Immunocytochemistry, ELISAs, Electrophysiological arrays</td>
<td>Difficulties in determination of acceptable variation values for each marker expression between different cell batches</td>
</tr>
</tbody>
</table>

We have listed here the current achievements and hurdles related to cell-manufacturing processes, differentiation, characterization of the cell products and preclinical animal studies of the cells.

FACS: Fluorescence-activated cell sorting; FP: Floor plate; GMP: Good manufacturing practice; hiPSC: human-induced pluripotent stem cell; HLA: Human leucocyte antigen; MACS: Magnetic activated cell sorting; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD: Parkinson’s disease; PET: Positron emission tomography; PGD: Preimplantation genetic diagnosis; qRT-PCR: Quantitative Reverse-Transcriptase Polymerase Chain Reaction; VM: Ventral midbrain; 6-OHDA: 6-hydroxydopamine.
contaminations or rejection events after transplantation. To gain totally xeno-free pluripotent stem-cell lines, several research groups have reported the removal of animal components from the inner cell mass isolation procedure [100]. There are also several commercial xeno-free culturing mediums for the maintenance and propagation of undifferentiated pluripotent stem cells, like mTESR [97] or E8 [99]. In the defined E8 medium, the basic DMEM/F12 includes only eight known factors that are most essential for survival and proliferation of pluripotent stem cells: insulin, selenium, transferrin, L-ascorbic acid, FGF2 and TGFβ in DMEM/F12 with pH adjusted with NaHCO₃. These defined factors are easily quality controlled, which also increases the safety of the stem-cell products and facilitates the transfer of these cells to clinical use. To date, there exist several 'clinical-grade' stem-cell lines that have been produced under GMP conditions and according to established guidelines and safety regulations to produce safe cell material for clinical transplantations [100-103].

8.5 Functionality and large-scale cell production of pluripotent stem-cell-derived DA neurons for clinical use

Current advances in the stem-cell field have made it possible to produce hiPSC-derived DA neurons for clinical use. Toward this end, it is important to have well-defined, simple and safe cell production protocols that produce an adequate number of cells for transplantation to PD patients. Previously, it has been shown that hESC- or hiPSC-derived VM DA neurons can improve motor deficits in PD-model rodents, and it has been shown that hESC- or hiPSC-derived VM DA neurons can improve motor deficits in PD-model rodents, and it could be postulated that stem-cell-derived DA neurons
may have similar capacities in clinical settings. In addition, these studies have also shown that the premature neuronal cells derived from pluripotent stem cells mature after transplantation in vivo with the support of surrounding cues from the host brain cells. This is important, since several studies have shown lack of terminal maturation of pluripotent stem-cell–derived cells in vitro, for example, difficulties in differentiation of functional DA neurons, hepatocytes or cardiomyocytes in vitro. Other problems related to scalability of cell production are the usually used two-dimensional (2D) culturing platforms that consist originally of feeder cells or Matrigel with the differentiating cells grown on top of them. These 2D systems require sequential passaging of the cells, numerous cell countings for replating sufficient concentrations of the differentiating cells, and daily medium changes. To increase efficacy and diminish variability in culturing conditions in the future, the scalability of cell production can be accomplished in 3D culturing systems, where the cells are grown in spheres in suspension. These 3D suspension cultures could be transferrable to bioreactors, where gas flows, medium change and cell passaging are automated and performed in a monitored environment [84]. Also, suspension culturing does not require xenogenic components such as mouse feeder cells or Matrigel, and xenogenic serum components can be replaced with defined medium components. The neural induction is also efficient in spheres, and the tight cell-to-cell contacts will facilitate efficient signaling and growth factor translation between differentiating neural precursors. This will potentially facilitate translating cell culturing to GMP and the establishment of large-scale cell production.

9. Conclusions

In this review, we have described the derivation of DA neurons from human pluripotent stem cells. We have also discussed the importance of characterization of the differentiated cell populations and identification of different subpopulations prior to transplantation. To assure the safety of the differentiated cell populations, it will be important to use a combination of surface molecule markers for enrichment and sorting of specific VM DA neuron cell populations. To avoid graft-induced side effects, the tumorigenic and serotonergic cells should be eliminated from the differentiated cell populations. Also, preclinical safety and efficacy testing should be performed in both rodent and nonhuman primate PD animal models. Nonhuman primate disease models are more comparable to human conditions than rodent models, since they allow prolonged follow-up times and enable comprehensive behavioral assays. For future clinical trials, establishment of HLA-matched hESC and hiPSC-line banks in GMP conditions will reduce the risk for immune rejection upon transplantation of pluripotent stem-cell–derived DA neurons. Further optimization of the currently existing differentiation protocols to GMP level with defined xeno-free culture conditions will increase the safety and feasibility of the cell products for human patients.

10. Expert opinion

At the moment, there exist several differentiation protocols to derive FP precursors from pluripotent stem cells, which can be further directed toward neural lineage and specific VM DA neurons [11,12,16,18,30,45]. However, most of these differentiation protocols for pluripotent stem-cell–derived neural cells still contain animal-derived substances and result in heterogeneous neural cell populations [11,12,18,30]. To overcome these problems, the materials used for neural differentiation can be converted to xeno-free products, and defined small molecules and xeno-free hydrogels could be used instead of recombinant animal-derived proteins and animal-protein matrices. Currently, most of these xeno-free factors and medium components are available from several vendors and new defined mediums have also been developed in several laboratories [99]. Also, the use of cell-surface–based cell-sorting methods could increase the safety of the cell grafts and make the cell populations more homogeneous and defined for transplantations. Currently existing sorting methods, for example, FACS and MACS, are reliable and safe methods to purify the populations; the limiting factor in these methods, however, is the lack of specific cell-surface markers for VM DA neuron precursors. The proper characterization of the cell populations with large patterns of surface markers will facilitate the characterization of specific markers for VM DA neuron cell sorting. In addition, after derivation of DA neurons from pluripotent stem cells, it is really important to establish variation limits for the expression levels of each marker studied. These established phenotypic characterization patterns of the cells will assure the proper identity and homogeneity of the cell batches after differentiation, diminish the variability between different cell batches prior to clinical use and could be a robust method in addition to bioassays involving transplantation of differentiated cells to rodent models.

For future large-scale cell production, the possibility of using suspension culturing for stem-cell–derived DA neuron differentiation would enable the transfer of these cells for bioreactor cultivation, where gas flows and medium changes are performed in controlled conditions under computer monitoring [84]. In addition, developed cryopreservation techniques will make it possible to establish cell banks for quality-checked clinical products [84,104]. The challenge in the current cell-banking approaches is to determine the right timing for freezing down the differentiated cells. The most optimal time for cryopreservation of the cells is probably immediately after they have been induced to FP precursors that express VM-type DA neural precursor markers like FOXA2/ NURR1 and FOXA2/LMX1A. After thawing the cells, the challenges remain in the establishment of sufficient recovery time for the cells before transplanting them to the patient’s brain. The optimal culturing time prior to transplantation
would probably vary by a couple of days, since cell death increases after thawing. Also, the addition of small molecules that can specifically block the cell-death pathways, like Rho-associated protein kinase ROCK inhibitor, can improve the recovery of the cells after thawing. In our opinion, the establishment of standard operating procedures for a simple, scalable cell preparation protocol, with defined factors, purification processes and cryopreservation techniques is the most important step to facilitate the translation of these cells safely for clinical use.

Because of the irreversible aspect of cell transplantation into the patient brain, the safety of the pluripotent stem-cell-derived cell grafts is crucial. That is why comprehensive and professionally executed preclinical animal studies for pluripotent stem-cell-derived DA neurons are important. The preclinical animal studies should assess the safety and functionality of the cell grafts with sufficient follow-up time. We think that both rodent and nonhuman primates are possible. However, nonhuman primates are the ideal models for cell transplantation mechanistic studies because of the physiological and behavioral similarities to humans and the scalability of the cell preparations. After transplantation of DA neurons, functional brain imaging with PET scanning can be used to detect the dopamine-releasing cell grafts in the primates. Comprehensive immune rejection studies should also be performed by staining T-lymphocytes and microglia around the graft site and by studying the amounts of reactive lymphocytes in the blood samples of the animals prior to and after transplantation [105]. In addition, it is important to establish a proper immunosuppression medication for xenografting human cells to monkeys, since this will also facilitate the selection of proper medication for human patients in case of hESC- or hiPSC-derived neural allografts. Overall, the long-term usage of immunosuppression medication is harmful for the recipients due to the decreased immune defense system against common diseases.

The previous problems related to graft rejection and the adverse effects of the use of immunosuppression medication could be bypassed with a proper selection of pluripotent stem-cell lines for neuronal differentiation and clinical translation. This means that if autologous hiPSC can not be obtained, then both the donor’s and recipient’s MHC/HLA classes should be matched with each other, with only minimal number (one or two) of mismatch alleles accepted. This is important, although the brain is considered to be an immune privileged site, because the allogenic iPSC-derived neural cell grafts can induce long-term activation of immune responses by activation of the microglia and infiltration of the leukocytes to the graft site. Since there is also variability in the neural differentiation capacity of different pluripotent stem-cell lines, we think that it will be important to select the cell lines that are more prone to produce VM DA neurons, according to preclinical characterization. The characterizations of the cells according to GMP regulations should cover the sterility, identity, safety and efficacy, stability, purity, and proliferation and differentiation capacity of the transplantable cells both in vitro and in vivo. After accomplishing all these safety and manufacturing requirements, human pluripotent stem-cell-derived DA neurons are usable for the establishment of a cell therapy option for PD patients. Although several clinical studies have shown the beneficial effects of human fetal VM DA neuron grafts in PD patients, the first clinical trials with pluripotent stem-cell-derived DA neurons should focus on the safety aspect of the cells. Due to the existing risk for tumorigenesis of pluripotent stem-cell-derived grafts, the first trials should be conducted with small doses of the cells and prove the phenotypical stability of the graft. Importantly, the cells should not cause any harm or adverse effects for the patients in long-term studies. After assuring the safety of the cells, the next step will be increasing the dosage of the transplantable cells for functional and efficacy studies of pluripotent stem-cell-derived DA neurons. These precautions are important to assure the safety of the patients who are participating in the clinical trials. Taking into account the rapid development of the stem-cell field and technological improvements in cell preparations and GMP facilities, we think that pluripotent stem-cell-derived DA neurons will offer a relevant cell therapy option for treatment of PD in the near future.

Declaration of interest

The authors have received funding from the National Institute of Health/National Institute of Neurological Disorders and Stroke, The Harvard Stem Cell Institute E Miller Translational Neuroscience Fund; Harold and Ronna Cooper family, the Consolidated Anti-Aging Foundation and the Poul Hansen family. No funding was received in support of this manuscript and the authors have no other competing interests to declare.
Bibliography

Papers of special note have been highlighted as either of interest (*) or of considerable interest (**) to readers.


of Parkinson’s disease. Stem Cells 2008;26:1526-36


98. Heng BC, Li J, Chen AK, et al. Translating human embryonic stem cells from 2-dimensional to 3-dimensional cultures in a defined medium on


Affiliation
Maria Sundberg PhD & Ole Isacson MD
†Author for correspondence
Neuroregeneration Research Institute, McLean Hospital/Harvard Medical School, 115 Mill St, Belmont, MA 02478, USA
Tel: +1 617 855 3283; Fax: +1 617 855 3284; E-mail: isacson@hms.harvard.edu