Induced pluripotent stem cells and Parkinson's disease: modelling and treatment

Xiaoyun Xu^{*1}, Jinsha Huang^{*1}, Jie Li^{*}, Ling Liu^{*}, Chao Han^{*}, Yan Shen^{*}, Guoxin Zhang^{*}, Haiyang Jiang^{*}, Zhicheng Lin[†], Nian Xiong^{*} and Tao Wang^{*}

^{*}Department of Neurology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China and

[†]Department of Psychiatry, Harvard Medical School, Division of Alcohol and Drug Abuse, Mailman Neuroscience Research Center, McLean Hospital, Belmont, MA, USA

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Abstract

Many neurodegenerative disorders, such as Parkinson's disease (PD), are characterized by progressive neuronal loss in different regions of the central nervous system, contributing to brain dysfunction in the relevant patients. Stem cell therapy holds great promise for PD patients, including with foetal ventral mesencephalic cells, human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs). Moreover, stem cells can be used to model neurodegenerative diseases in order to screen potential medication and explore their mechanisms of disease. However, related ethical issues, immunological rejection and lack of canonical grafting protocols limit common clinical use of stem cells. iPSCs, derived from reprogrammed somatic cells, provide new hope for cell replacement therapy. In this review, recent development in stem cell treatment for PD, using hiPSCs, as well as the potential value of hiPSCs in modelling for PD, have been summarized for application of iPSCs technology to clinical translation for PD treatment.

Abbreviations

adeno-iPSCs: adenoviral iPSCs

CD: cluster of differentiation

CM: cynomolgus macaque

DA: dopamine

DAT: dopamine transporter

DBS: deep brain stimulation

FACS: flow cytometric analysis

GBA: glucocerebrosidase

hESCs: human embryonic stem cells

hiPSCs: human induced pluripotent stem cells

LBs: Lewy bodies

LRRK2: leucine-rich repeat kinase 2

MEF: mouse embryonic fibroblasts

MHC: major histocompatibility complex

mtDNA: mitochondrial DNA

NSCs: neural stem cells

PD: Parkinson's disease

SGZ: subgranular zone

SNCA: α -synuclein

SNpc: substantia nigra pars compacta

SVZ: subventricular zone

VPA: valproic acid

ZFN: zinc finger nuclease

ZNS: zonisamide

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, and concerns progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) of the midbrain (1). The

Correspondence: T. Wang, Department of Neurology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Road, Wuhan 430022, Hubei, China;

E-mail: wangtaowh@hust.edu.cn

¹These authors contributed equally to this work.

crucial pathological feature of PD is presence of Lewy bodies (LBs), which are abnormal aggregates of α -synuclein (SNCA) protein. Reported standardized incidence rates of PD are 8–18 per 100 000 person-years worldwide (2). In China, prevalence of PD for those aged ≥ 65 years is 1.7% (3). PD patients suffer from motor symptoms such as rest tremor, bradykinesia, rigidity and abnormal gait. Non-motor symptoms, such as olfactory dysfunction, psychiatric changes and sleep disorders, further impair PD patients' quality of life. Up to now, multiple factors have been found to involve pathogenesis of PD, including genetic susceptibility, environmental toxins, interruption of autophagy, neuroinflammation and most importantly – advancing age. Although the precise mechanisms underlying the pathogenesis of PD are not well understood, interactions within these pathogenic factors give rise to loss of DA neurons within the SNpc. Unfortunately, current pharmacological and surgical treatments provide only insufficient symptomatic relief, but cannot reverse nor slow down the underlying loss of midbrain DA neurons. Stem cell transplantation, however, holds great promise in the treatment of PD.

Neural stem cells (NSCs) provide a potential endogenous source for neuron replacement therapy in neurodegenerative disorders such as PD. One of the most important essential features of NSCs is their proliferation potential. It has already been indicated that NSCs can differentiate directly into DA neurons and Suksuphew and Noisa have shown that they have high possibility for producing two undifferentiated daughter cells at early stages of development (symmetric division), and later cell division for production of differentiated neurons plus glial cells (asymmetric division) (4). A further feature of NSCs' multipotency is their potential to differentiate into astrocytes and oligodendrocytes, as well as into neurons (5,6). NSCs in the subventricular zone (SVZ) can differentiate into olfactory neurons, while those of the subgranular zone (SGZ) can differentiate into granular neurons of the dentate gyrus (7). Furthermore, when implanted into developing eyes, hippocampal NSCs have exhibited several morphological and immunological properties of retinal cells, including photoreceptors (7). Differentiation of adult NSCs can be influenced by their local environment as well as by intrinsic programmes (8).

Human ESCs were the first human stem cells to be identified and cultured, by Thomson *et al.* in 1998 (9), and were proven at that time to be self-renewing and pluripotent. These properties indicated hESCs as having great promise for cell transplantation therapy. However, ethical concerns arose immediately as generation of hESCs requires destruction of the fertilized human embryo. A further significant problem with transplanting

stem cells is associated with immunological rejection after transplantation of specific cells derived from allogeneic hESCs. In 2006, Yamanaka *et al.* reported generation of ES-like pluripotent stem cells from somatic fibroblasts, the so-called iPSCs (10). Since then, many methods have been explored to generate hiPSCs from a wide variety of easily accessible source tissues, including skin, adipose and blood cells (11–14). Unlike hESCs, there are no ethical issues preventing use of iPSCs. Refinement of reprogramming methods now allows for iPSC generation without genomic integration of reprogramming factors, using expression plasmids, non-integrating viruses, recombinant proteins, small molecules and synthetically modified mRNAs or miRNAs (15). Here, we review the existing iPSC-based models and treatments, with particular emphasis on PD, and explore the challenges associated with cell therapies using iPSCs-derived DA neurons, which have thus far hindered expansion of this research.

The long journey to optimize a iPSC-derived DA neuron differentiation protocol

Cell replacement therapy with foetal ventral midbrain (VM) DA neurons has been shown, in some ways, to be beneficial to PD patients. Dopaminergic neurons lost in PD are primarily of the VM, and VM DA neurons arise from floor plate cells during embryonic development. The earliest study of DA neuron differentiation from mouse ESCs was performed by Lee *et al.* in 2000 (16). This group generated CNS progenitor populations from ESCs, expanded the cells and promoted their differentiation into dopaminergic and serotonergic neurons in the presence of mitogens and specific signalling molecules. Their differentiation involved a number of steps: generation of embryoid bodies (EBs) without retinoic acid (RA) treatment in a serum-containing medium, use of a defined medium to select for CNS stem cells, proliferation of CNS stem cells in the presence of mitogen, basic fibroblast growth factor (bFGF), and differentiation of the stem cells by removal of the mitogen in serum-free medium (16). Finally, the differentiation medium consisted of N2 medium supplemented with laminin, cAMP and ascorbic acid (AA). Sonic hedgehog (SHH), FGF8 and AA also enhanced differentiation to DA fate, and increased yield of ES-derived TH⁺ neurons. The cells were incubated under differentiation conditions for 6–15 days at the last stage, in order to increase numbers of TH⁺ neurons and DA level (16). While this protocol succeeded in generating DA neurons at relatively high efficiency, extensive studies in Parkinsonian animals are needed to further assess complete function and safety of ESC-derived DA neurons *in vivo* (16). Efficiency and

purification of generated cell populations also needs to be improved by genetic methods.

After some months, Kawasaki and colleagues introduced an efficient method for generating neurons from ESCs by using PA6-derived stromal cell-derived inducing activity (SDIA) in a serum-free condition requiring neither EBs nor RA treatment (17). High proportions of TH⁺ neurons producing DA were obtained from SDIA-treated ESCs. When transplanted, SDIA-induced dopaminergic neurons integrated into the mouse striatum and remained positive for TH expression (17). In accordance with Lee's group, Kawasaki *et al.* also avoided using RA in their experiment, as RA seemed to perturb neural patterning and neuronal identities in EBs, as a strong teratogen. Efficiency of DA neuron induction in the SDIA method is as high as maximum efficiency (~30%) obtained by Lee's method with SHH, FGF8 and ascorbate treatment (17). Neural induction by SDIA provided a new powerful tool for both basic neuroscience research and therapeutic applications.

Low efficiency of generation of DA neurons from primary cultures of foetal neonatal cells, or adult stem

cells, limits their therapeutic potential as donor cells (18). In effort to improve efficiency of DA neuron generation, survival and maturation *in vitro*, Kim *et al.* used a cytomegalovirus plasmid (pCMV) driving expression of rat Nurr1 complementary DNA modified to establish stable Nurr1 ESC lines (18). Nurr1 ESCs raised the proportion of TH⁺ neurons up to 78%, combined with their previous five-stage differentiation method (16,18). They also demonstrated that these DA neurons from ESCs could functionally integrate into host tissue as well as lead to recovery in a rodent model of PD (18).

These results have subsequently been replicated using hESCs with some modifications, but efficiency was not satisfactory (19–21). Perrier *et al.* have reported that co-culture of hESCs on MS5 stroma can yield highly efficient differentiation into midbrain DA neurons (22). Neural differentiation of hESCs was induced by means of co-culture on MS5, MS5-Wnt or S2 stroma at comparable efficiencies. Growth factors were added in various combinations and at various time points. Rosette structures were harvested mechanically from feeder layers on day 28 of differentiation and gently replanted on

Table 1. Summary of publications using iPSCs for modelling PD with gene mutations

Gene	Mutations	Findings	Publications
PARK2	Exon 3/5 deletion	Increased the transcription of monoamine oxidases, oxidative stress and spontaneous DA release, reduced DA uptake	Jiang <i>et al.</i> (53)
	Exon 2-4 and exon 6,7 deletions	Increased oxidative stress, impaired mitochondrial homoeostasis and SNCA accumulation	Imaizumi <i>et al.</i> (54).
	Exon 3,5 deletions	Decreased complexity of neuronal processes and microtubule stability	Ren <i>et al.</i> (55)
	R42P EX3DEL R275W Q456X	Decreased DA differentiation, accompanied by reduced mitochondrial volume ratio and abnormal mitochondrial ultrastructure More sensitive to cell death and production of ROS elicited by mitochondrial and oxidative stressors, and increased basal oxygen consumption and proton leakage Phenotype rescue using coenzyme Q10, rapamycin or the LRRK2 kinase inhibitor GW5074	Shaltouki <i>et al.</i> (48). Cooper <i>et al.</i> (57)
LRRK2	G2019S	Increased expression of key oxidative stress-response genes and α -synuclein More sensitive to caspase-3 activation and cell death caused by exposure to stress agents	Nguyen <i>et al.</i> (58)
	G2019S, R1441C	Reduced the availability of substrates for oxidative phosphorylation, and disrupted mitochondrial movement	Cooper <i>et al.</i> (57)
	G2019S, R1441C	Induced mitochondrial DNA damage, and this phenotype can be functionally reversed or prevented by zinc finger nuclease (ZFN)-mediated genome editing	Sanders <i>et al.</i> (59)
SNCA	Triplication	Accumulation of SNCA, overexpression of markers of oxidative stress, and sensitivity to peroxide induced oxidative stress	Byers <i>et al.</i> (60)
GBA1	A53T	Increased nitric oxide and 3-NT levels	Chung <i>et al.</i> (61)
	L444P, N370S	Reduced glucocerebrosidase activity and protein levels, increased α -synuclein levels as well as autophagic and lysosomal defects Dysregulation of calcium homoeostasis and increased vulnerability to stress responses involving elevation of cytosolic calcium	Schondorf <i>et al.</i> (63)
	N370S	Impaired SNCA clearance regardless of disease status; non-genetic factors further perturb DA homoeostasis in addition to <i>GBA</i> mutations	Woodard <i>et al.</i> (64)

15 µg/ml polyornithine/1 µg/ml laminin-coated culture dishes in N2 medium supplemented with SHH, FGF8, AA and BDNF. These cells were resuspended in N2 medium, and replated again on to polyornithine/laminin-coated culture dishes in the presence of SHH, FGF8, AA and BDNF. After additional 7–9 days culture, cells were found to have differentiated in the absence of SHH and FGF8 but in the presence of BDNF, glial cell line-derived neurotrophic factor, transforming growth factor type β 3, dibutyryl cAMP and AA (22). The workers observed that exposure to SHH and FGF8 from day 12 to day 20 differentiation, followed by differentiation in the presence of AA and BDNF, resulted in 3-fold increase in TH⁺ cells. Up to 79% of all the neurons express TH, the rate-limiting enzyme in the synthesis of DA. In addition to TH expression, cells in these cultures expressed key markers associated with normal midbrain DA neurons. However, the high-yield midbrain DA neuron derivation protocol reported here need to be transplanted into pre-clinical animal models of PD (22). Beyond this, cell survival and long-term maintenance of phenotype are essential parameters for testing *in vivo*.

In 2008, a group of Korean scientists reported a method for differentiating hESCs into functional TH⁺ neurons, with up to near 86% total hESC-derived neurons, the highest purity ever reported (23). The unique feature of their protocol was generation of pure spherical neural masses (SNMs). These SNMs could be expanded for long periods without losing their differentiation capability and could be coaxed into DA neurons efficiently within a relatively short time (approximately 2 weeks) when needed. SNM culture and DA neuron derivation from the SNMs did not need feeder cells, which reduced risks of contamination by unwanted cells and pathogens. More importantly, their hESC-derived DA neurons induced clear behavioural recovery after transplantation in a Parkinsonian rat model, indicating their functionality *in vivo* (23).

It has been reported that bone marrow mesenchymal stem cells (BMSCs) can differentiate into not only osteogenic, adipogenic, chondrogenic cells, but also into other lineages including myogenic, hepatic and neurogenic cells (24). Furthermore, they are inducible to differentiate into cells with the DA neuronal phenotype suggested by expression of TH, DAT markers, as well as synthesis and secretion of DA after appropriate stimuli (25). Previous studies have shown that human BMSC engraftment can alleviate motor dysfunction in Parkinsonian animal models, but with limited efficacy and but few engrafted cells surviving. Our team transplanted equal amounts of hBMSCs into hemi-lesioned Parkinsonian rats with supplementation of bFGF, to assess whether a combination of bFGF and hBMSC

therapy would enhance treatment effectiveness in PD rat models (26). As a result, bFGF promoted hBMSCs to transdifferentiate towards neural-like lineages *in vitro* (26). In addition, hBMSC transplantation alleviated motor functional asymmetry, and prevented DA neurons from loss in the PD model, while bFGF administration enhanced neurodifferentiation capacity and therapeutic effect (26).

Similar strategies have been applied for differentiating hiPSCs into DA neurons. Cooper *et al.* postulated that a major limitation for experimental studies of current ESC/iPSC differentiation protocols, was lack of VM DA neurons of stable phenotype, as defined by expression marker code FOXA2/TH/ β -tubulin (27). They demonstrated a combination of three modifications that were required to produce VM DA neurons. First, early and specific exposure to low-dose RA improved regional identity of neural progenitor cells derived from pluripotent stem cells. Secondly, a high activity form of human SHH established a sizeable FOXA2⁺ neural progenitor cell population *in vitro*. Thirdly, early exposure to FGF8a, rather than FGF8b, and WNT1 were required for robust differentiation of the FOXA2⁺ floor plate-like human neural progenitor cells into FOXA2⁺ DA neurons (27). FOXA2⁺ DA neurons were also generated when this protocol was adapted to feeder-free conditions. In summary, their new human ESC and iPSC differentiation protocol can generate human VM DA neurons as required for relevant new bioassays, drug discovery and cell-based therapies for PD (27).

iPSC: for Parkinson's disease modelling

The majority of PD cases are sporadic with unknown cause. Age, oxidative stress, toxin and environmental factors are risk factors (2), and remaining 10% is familial PD, where several causative genes have been identified (28). Before stem cell modelling appeared, the most used cell or animal models for PD were generated with toxins such as rotenone, 6-OHDA, MPTP or genetic models. The relationship between MPTP and PD was found in a cluster of young drug addicts, by Davis *et al.* in 1979 (29). MPTP easily crosses the blood–brain barrier (BBB) where it is oxidized in glial cells into MPP⁺. MPP⁺ competes with DA for the DA transporter and after entering neurons, it exerts its toxic effect by inactivating complex I of the ETC (30). MPTP is commonly used to model for PD in primates and rodents in that the drug kills dopaminergic neurons, allowing researchers to study neuronal circuitry with reduced dopaminergic involvement (31). Many workers have demonstrated that MPTP administration is able to reproduce most, but not all, the clinical and pathological hallmarks of PD in

Table 2. Summary of publications using iPSCs for treating PD

Cell sources	Subjects	Effectiveness	Publications
DA neurons derived from directly reprogrammed fibroblasts	6-OHDA-lesioned rats	A large number of TH-positive cells were observed in the striatum, which were also positive for En1, VMAT2 and DAT; animals showed a marked recovery of the rotation behaviour 4 weeks after transplantation	Wernig <i>et al.</i> (78)
DA neurons derived from hiPSCs	MPTP-lesioned monkeys	Grafted neurons survived as DA neurons as long as 6 months	Kikuchi <i>et al.</i> (80)
iPSCs from Cynomolgus macaque skin fibroblasts carrying specific major histocompatibility complex haplotypes	6-OHDA-lesioned rats	Neither tumour formation nor inflammatory reactions happened in the transplanted animals up to 6 months after transplantation	Deleidi <i>et al.</i> (81)
CORIN+ hiPSC-derived DA progenitor cells	6-OHDA-lesioned rats	The CORIN+ cells survived and differentiated into mDA neurons <i>in vivo</i> , significant improvement of the motor behaviour, without tumour formation	Doi <i>et al.</i> (85)
cynomolgus monkey iPSC-derived midbrain DA neurons	MPTP-lesioned cynomolgus monkey (CM)	A gradual onset of functional motor improvement, and increased motor activity, without a need for immunosuppression; robust survival of midbrain-like DA neurons and extensive outgrowth into the transplanted putamen	Hallett <i>et al.</i> (86)

monkeys (32–34) and, at least degeneration of dopaminergic neurons, in mice (35). Similar to MPTP, the pesticide rotenone disrupts complex I function of mitochondria (36). Our team has demonstrated that rotenone models for PD appear to mimic most clinical features of idiopathic PD and recapitulate the slow and progressive loss of DA neurons and LB formation in the nigral-striatal system (36). Both MPTP and rotenone have been important for establishment of PD animal models. However, while they promote dopaminergic neuron death with associated motor impairment, their side effects and lack of specificity are major drawbacks (31). 6-OHDA, a selective catecholaminergic neurotoxin, is used to generate lesions in the nigrostriatal DA neurons in rats (37). Unlike MPTP, 6-OHDA cannot cross the BBB. So, 6-OHDA must be injected into the SNc, medial forebrain bundle or striatum, to induce Parkinsonism, rather than systemic administration (38). Intrastriatal injection of 6-OHDA causes progressive retrograde neuronal degeneration in the SNc and VTA (39,40). Genetic models provide us with better understanding of underlying genetic forms of PD, even though their pathological and behavioural phenotypes are often quite different from the human condition (41). Many genetic variant models, including *SNCA*, *LRRK2*, *PINK*, *Parkin*, *DJ-1* and Glucocerebrosidase (*GBA*), have been generated to explore inherent mechanisms in

PD (42–45). The well-established genetic models are able to interpret pathogenesis of only 5–10% familial PD, however, without replicating the entire genetic background of the patients *in vitro* (46). Moreover, differences between species in displaying neurodegenerative phenotypes make it difficult to extrapolate results obtained from animal models to humans (47). The discovery of iPSCs has for the first time enabled us to reproduce DA neurons from individuals who suffered from familial or sporadic PD (47,48). Moreover, these iPSC models allow us to explore pathogenic factors and discover interactions between genetic and exogenous factors involved in the pathogenesis of PD. As individuals' responses to drug compounds varies, patient-specific iPSCs may be used to distinguish between those individuals likely to respond to new therapeutics and those who are not, and more accurately predict toxicity and efficacy in screening drugs, from mechanisms, in comparison to animal models.

Differentiation of DA neurons from iPSCs has been demonstrated to be relatively robust and reproducible, allowing for generation of disease models from patients carrying a variety of mutations in key genes implicated in familial PD, including *PARK2*, *PINK1*, *LRRK2*, *SNCA* and *GBA* (49,50). Among genetic risk factors, *Parkin* (*PARK2*) is the most frequently mutated gene that has causally been linked to autosomal recessive

early-onset familial PD (51). In patients with PD onset before the age 45, *PARK2* mutations are seen in up to 50% of familial cases and about 15% of sporadic cases (52). *Parkin* knockout mouse models display some abnormalities, but do not fully recapitulate the pathophysiology of human *PARK2*. Jiang *et al.* generated iPSCs from normal subjects and PD patients with *Parkin* mutations and demonstrated that loss of *Parkin* in human midbrain DA neurons greatly increased expression of monoamine oxidases and oxidative stress, significantly reduced DA uptake and increased spontaneous DA release (53). These results suggest that *Parkin* controls dopamine utilization in human midbrain DA neurons by enhancing the precision of DA neurotransmission and suppressing DA oxidation (53). Imaizumi *et al.* used *PARK2* patients' specific iPSCs-derived neurons to recapitulate pathogenic changes in the brain of *PARK2* patients (54). The data indicated that *PARK2* iPSC-derived neurons exhibited increased oxidative stress, impaired mitochondrial homeostasis and SNCA accumulation (54). Recently, Ren *et al.* showed that the complexity of neuronal processes and microtubule stability were significantly reduced in iPSC-derived neurons from PD patients with *Parkin* mutations (55), suggesting that *Parkin* maintains morphological complexity of human neurons by stabilizing microtubules (55). Shaltouki *et al.* observed reduced DA differentiation, accompanied by reduced mitochondrial volume ratio and abnormal mitochondrial ultrastructure, consistent with the current model of *PARK2* mutations (48).

PINK1 functions upstream of *Parkin*, and is involved in recruiting *Parkin* to damaged mitochondria, for example, following mitochondrial depolarization (56). Mutations in either *PARK2* or *PINK1* result in impaired mitophagy. Cooper *et al.* found that *PINK1* mutant iPSC-derived DA neuronal cells were more sensitive to cell death and production of ROS elicited by mitochondrial and oxidative stressors, and further showed increased basal oxygen consumption and proton leakage suggestive of intrinsically damaged mitochondria (57). Moreover, cell vulnerability associated with mitochondrial function in iPSC-derived neural cells could be rescued with coenzyme Q10, rapamycin or LRRK2 kinase inhibitor GW5074. These data demonstrate that iPSC-derived neural cells are sensitive models for measuring vulnerability and dose-responses of candidate neuroprotective molecules and might help to identify disease causes and better individualize treatment efficacy (57).

Mutations in leucine-rich repeat kinase 2 (*LRRK2*) are associated with sporadic and familial forms of PD. Nguyen *et al.* have reported that DA neurons derived from G2019S mutation-iPSCs have high levels of

expressions of key oxidative stress-response genes and SNCA protein (58). The mutant neurons were more sensitive to caspase-3 activation and cell death caused by exposure to stress agents, such as hydrogen peroxide, MG-132 and 6-hydroxydopamine (58). Cooper *et al.* indicated that *LRRK2* G2019S and R1441C mutations reduced availability of substrates for oxidative phosphorylation, and were associated with disrupted mitochondrial movement in PD patient-specific iPSCs (57). Laurie *et al.* further demonstrated the mechanisms by which *LRRK2* mutations lead to loss of mitochondrial function. Their data revealed that mitochondrial DNA (mtDNA) damage was induced in neural cells by PD-associated mutations in *LRRK2*, and this phenotype could be functionally reversed or prevented by zinc finger nuclease (ZFN)-mediated genome editing in iPSCs (59). These results indicate that mtDNA damage is likely to be a critical early event in neuronal dysfunction that leads ultimately to *LRRK2*-related PD (59).

The first genetic cause identified for familial PD was SNCA, as PD can be caused by mutations in SNCA or by overexpression of normal SNCA via gene duplication or triplication, consistent with a gain-of-function mechanism. iPSC-derived midbrain DA cultures from SNCA triplication patients exhibit several disease-related phenotypes in culture, including accumulation of SNCA, inherent overexpression of markers of oxidative stress and sensitivity to peroxide-induced oxidative stress (60). iPSCs, reprogrammed from patients with the most common A53T-SNCA mutation, had high nitric oxide and 3-NT levels compared to controls (61).

GBA mutations, which cause the lysosomal storage disorder Gaucher disease, have recently been linked to a 5-fold greater risk of developing Parkinsonism than non-carrier individuals (62), and are the strongest genetic risk factor for PD known to date. *GBA1* mutated iPSC-derived neurons have low glucocerebrosidase activity and protein levels, and high SNCA levels as well as autophagic and lysosomal defects (63). Mutant neurons display dysregulation of calcium homeostasis and increased vulnerability to stress responses involving elevation of cytosolic calcium (63). These findings using iPSC technology, have provided evidence for a link between *GBA1* mutations and complex changes in autophagic/lysosomal system and intracellular calcium homeostasis, underlying vulnerability to neurodegeneration. As monozygotic twins share identical genetic makeup, twin studies have been valuable for dissecting complex gene-environmental interactions in PD. Woodard *et al.*, using iPSC technology (64), investigated a unique set of monozygotic twins and found that SNCA clearance was impaired in midbrain DA neurons carrying *GBA* N370S regardless of disease status. Moreover, DA

levels of twins discordant for PD were different, suggesting that non-genetic factors further perturbed DA homeostasis in addition to *GBA* mutations. These results verified the interactions between genetic and environmental factors in the progress of PD, and offer a theoretical basis for personalized medicine in PD (Table 1).

Although the cause of sporadic PD is not fully understood, various factors including environmental toxins, genetic susceptibility and age, have been implicated. Isogenic hiPSC PD models show that toxin-induced nitrosative/oxidative stress results in S-nitrosylation of transcription factor MEF2C and this redox reaction inhibits MEF2C-PGC1 α transcriptional network, contributing to mitochondrial dysfunction and apoptotic cell death (65), suggesting that the MEF2C-PGC1 α pathway may be a new drug target for PD. The advance of iPSC technology now enables widespread development of PD models for dissecting molecular mechanisms that contribute to its disease pathogenesis.

hiPSCs: for treating Parkinson's disease

As previously mentioned, there is currently no cure for PD except for some extent of relieving the symptoms. Current treatments include the use of oral medication of L-DOPA dopamine receptor agonists, MAO-B, apomorphine in more serious cases, continuous intestinal infusion of L-DOPA, and deep brain stimulation (DBS) in subthalamic nucleus and globus pallidus by using surgically implanted electrodes (66). L-DOPA is the gold standard for treatment of PD. Up to now, no medical nor surgical therapy has been shown to provide superior anti-parkinsonian benefits than can be achieved with L-DOPA (67). Unfortunately, its therapeutic effect is reduced after around 3–5 years use (68). The problems and limitations associated with long-term use of L-DOPA, including on-off fluctuations and emergence of dyskinesia, facilitating exploration of better ways to restore dopamine neurotransmission. Dopamine receptor agonists are used as the first choice to delay initiation of L-DOPA treatment, with longer plasma elimination half-lives than L-DOPA. Their mechanism of action are by stimulation of presynaptic and postsynaptic DA receptors so that their use has therefore been considered to be opportunity to improve continuous drug delivery (67). Selegiline was the first selective, irreversible inhibitor of monoamine oxidase type B (MAO-B) used in treatment of PD, which can stabilize DA levels in the synaptic cleft (67). Because of its capacity for interfering with oxidative stress and for blocking MPTP toxicity, selegiline has been tested in the first major trial as a putative disease-modifying agent (67). Rasagiline is another MAO-B inhibitor, with different metabolites than

selegiline, successfully developed for PD therapy. Good tolerance to rasagiline and its ease of use make it an appealing option at the start of therapy (67). DBS as surgical treatment has some serious limitations. It is costly and can produce cognitive disorders, which may be permanent (68). All of these treatments have considerable side effects such as ultimate loss of drug effect ('wearing off') during disease progression, occurrence of dyskinesia (notably with L-DOPA) use, and appearance of non-motor symptoms that are largely refractory to dopaminergic medication (69). The concept of using cell transplantation to substitute for loss of DA neurons in the brains of PD patients has evolved. In addition to conventional clinical treatments, such as pharmaceutical drugs and DBS, cell replacement therapy has offered a novel basis for development of effective therapeutic strategies for PD. In 1987, Brundin *et al.* first transplanted human VM tissue into the striatum of PD patients in Sweden, and the era of cell therapy for PD patients started (70). Various source tissues have been assessed for therapeutic replacement of DA neurons, such as hESC, hiPSC or DA grafts directly converted from somatic cells. Our team also has transplanted DiI-labelled human umbilical cord mesenchymal stem cells (HUMSCs) to rotenone-induced hemiparkinsonian rats (71). We showed that intra-CPu transplantation of DiI-labelled HUMSCs 4 weeks after rotenone administration ameliorated APO-induced rotations gradually over a period of 12 months, indicating long-term therapeutic effect of this approach (71). By monitoring red fluorescence of DiI, we found that HUMSCs migrated in the lesioned cerebral hemisphere, from CPu to SNc, or even to the opposite hemisphere through the corpus callosum. HUMSCs survived for up to 12 months after transplantation, and differentiated into Nestin-, NSE-, GFAP- and TH-positive cells in the CPu and TH⁺ cells in the SNc. No tumour-like structures was observed in implanted CPu (71). As reported, vascular endothelial growth factor (VEGF) is a neurotrophic factor which has been proven to promote growth and survival of DA neurons in VM explants and animal models for PD (72–74). Our previous work has also indicated that relatively low-level expression of VEGF in the striatum protects DA neurons of Parkinsonian rats (75). Next, we developed a more effective neurorestorative and neuroregenerative therapy combining VEGF and HUMSC (76). As a result, intrastriatal infusion VEGF-expressing HUMSCs to rotenone-induced Parkinsonian rats provided a significant behavioural improvement, more significant than HUMSC transplantation alone, and resulted in revival of TH immunoreactivity in the lesioned striatum and SNc (76). Importantly, VEGF expression enhanced neuroprotective effects by promoting DA neuron-orientated

differentiation of the HUMSCs. Thus, our findings have presented the suitability of HUMSC as a vector for gene therapy and suggested that stem cell engineering with VEGF may improve transplantation strategies for PD treatment (76).

iPSCs, induced pluripotent cells, have the potential capacity for self-renewal and are able to differentiate into any somatic cells, including DA neurons (77). Alternatively, iPSCs have properties similar to ESCs but can be generated from adult human cells such as skin, adipose tissue and fibroblasts (10). Thus, they are ethically more acceptable than some other stem cells sources. In theory, iPSCs from patients are without risk of immunological rejection for autografting (78).

Before successfully generating hiPSCs, many efforts had been made in animal experiments. In 2006, Yamanaka *et al.* generated iPSCs from mouse embryonic fibroblasts (MEF) and adult mouse tail-tip fibroblasts, by retrovirus-mediated transfection of four transcription factors, Oct3/4, Sox2, c-Myc and Klf4 (10). One year later, iPSCs were derived by the same group (79) by viral reprogramming of human skin fibroblasts with the same four factors. These studies opened a new avenue for generating patient- and disease-specific pluripotent stem cells. Wernig *et al.* used 6-OHDA-lesioned rats to examine whether DA neurons derived from directly reprogrammed fibroblasts had therapeutic potential for PD animals (78). As a result, in the striatum of rats grafted with differentiated iPSCs, a large number of TH⁺ cells with complex morphologies have been observed, these grafted stem cells were also positive for En1, VMAT2 and DAT. Four of the five transplanted animals which contained large numbers of TH⁺ neurons showed marked recovery of rotation behaviour 4 weeks after transplantation (78).

Kikuchi *et al.* first grafted hiPSC-derived DA neurons into the brains of an MPTP-lesioned Parkinsonian monkey, which survived as DA neurons as long as 6 months (80). In order to reduce immune rejection, Deleidi *et al.* generated iPSCs from cynomolgus macaque (CM) skin fibroblasts carrying specific major histocompatibility complex (MHC) haplotypes, observing that neither tumour formation nor inflammatory reactions occurred in the transplanted animals, up to 6 months after transplantation (81). Concerning the aspect of directed differentiation of iPSC into DA neurons, Sánchez-Danes *et al.* indicated lentiviral vectors driving controlled expression of LMX1A was an efficient way to generate enriched populations of human VM DA neurons (82). However, Mak *et al.* revealed that the protocol using dorsomorphin and SB431542 to replace SHH with purmorphamine or smoothened agonist could greatly improve conversion of hiPSCs to the neuronal

lineage (83). These histocompatible iPSCs may allow pre-clinical validation of safety and efficacy of iPSCs for PD.

Two commonly used anti-convulsants drugs, valproic acid (VPA) and zonisamide (ZNS), have been tested to promote differentiation of iPSC-derived DA neurons (84). As iPSC-derived donor cells inevitably contain tumourigenic or inappropriate cells, finding better protocols to purify and sort iPSCs is urgent. Doi *et al.* have shown that hiPSC-derived DA progenitor cells can be efficiently isolated by cell sorting using a floor plate marker, CORIN (85). When transplanted into 6-OHDA-lesioned rats, CORIN⁺ cells survived and differentiated into midbrain DA neurons *in vivo*, resulting in significant improvement in motor behaviour, without tumour formation (85). Recently, Hallett *et al.* analysed CM iPSC-derived midbrain DA neurons for up to 2 years following autologous transplantation in a PD model. They observed that unilateral engraftment of CM-iPSCs provided gradual onset of functional motor improvement, and increased motor activity, without any need for immunosuppression. Postmortem analyses demonstrated robust survival of midbrain-like DA neurons and extensive outgrowth into the transplanted putamen (86). These experiments offered strong immunological, functional and biological rationales for using midbrain DA neurons derived from iPSCs for future cell replacement in PD (Table 2).

Challenges for iPSC-derived cell therapies in Parkinson's disease

Although pre-clinical studies concerning iPSCs-derived cell therapies have shown great achievement, yet, some limitations hinder clinical usage of iPSCs for PD treatment. Tumourigenicity of iPSCs is an important putative problem. Murine iPSCs and ESCs both form teratomas when transplanted into syngeneic mice. Also, hiPSCs and hESCs generate teratomas when injected into immunodeficient mice (87). A standardized sensitive teratoma assay to detect low numbers of tumour-forming cells within a therapeutic cell preparation would be highly valuable. Gropp *et al.* presented detailed characterization of an efficient, quantitative, sensitive and easy-to-perform teratoma assay (87). These tumours may be benign (also, they may be malignant), although even so can become fatal when very large. In some studies, when teratomas have been removed from mice, the animals survived (88). Importantly, aggressiveness of teratocarcinomas from iPSCs is greater than that of ESCs (88). Differences in oncogenicity between ESCs and iPSCs might be due to their different approach of being derived (88). As hiPSCs have been first derived

by transduction of human dermal fibroblasts with integrating viruses carrying four transcription factors Oct4, Sox2, c-Myc and Klf4 (79), c-Myc is a well-established oncogene while the other three transcription factors are known to be highly expressed in various types of cancer (89–91). Yamanaka *et al.* subsequently reported a further Myc family member, L-Myc, as well as C-Myc mutants (W136E and dN2), all of which indicated little transformational activity, promoting hiPSC generation more efficiently and specifically compared to WT C-Myc (32). A further cause of tumorigenicity may be attributed to random integration of foreign DNA into the host genome disrupting important genes or activating oncogenes, potentially leading to uncontrollable growth of cells (92).

Stadtfield *et al.* generated mouse iPSCs from fibroblasts and liver cells by using non-integrating adenoviruses transiently expressing Oct4, Sox2, Klf4 and c-Myc (93). These adenoviral iPSCs (adeno-iPSCs) showed DNA demethylation characteristic of reprogrammed cells, expressed endogenous pluripotency genes and formed teratomas (93). Their work indicated that insertional mutagenesis was not required for *in vitro* reprogramming (93). Thus, more than 2 years after establishment of iPSC technology by Yamanaka's group, these newly generated adeno-iPSCs have been the first reported reprogrammed pluripotent stem cells with evidence of complete lack of viral transgene integration (94). Yamanaka *et al.* described an alternative method to generate iPSCs from MEFs by continual transfection of plasmid vectors free from plasmid integration (95); this protocol took around 2 months to complete, from MEF isolation to iPSC establishment. The virus-free technique reduced safety concerns for iPSC generation and application, and have provided a source of cells for investigation of mechanisms underlying reprogramming and pluripotency (95). Introducing mRNA directly into host cells without altering their genomic makeup or using episomal DNA-based vectors which seldom integrate into the host genome, holds the potential to solve this problem, by providing sufficient reprogramming factor expression for successful transformation of somatic cells (96–98).

Anokye-Danso *et al.* showed that expression of the miR302/367 cluster rapidly and efficiently reprogrammed mouse and human somatic cells to an iPSC state without requirement for exogenous transcription factors (99). This miRNA-based reprogramming approach was two orders of magnitude more efficient than standard Oct4/Sox2/Klf4/Myc-mediated methods, and the miR302/367 iPSCs displayed characteristics similar to the Oct4/Sox2/Klf4/Myc-iPSCs (99).

A further problem which hinders iPSCs treatment is that the therapeutic effect can be influenced by inherent pathogenic features of PD. Interactions between genetic and exogenous factors result in its pathogenesis. A general concern about use of autologous iPSC transplantation is whether underlying PD-associated genetic mutations presented in transplanted neurons increases vulnerability of iPSC-derived midbrain DA neurons to disease pathology. Environmental factors and age contribute largely to the pathogenesis of PD. Thus, iPSC-derived neurons represent a reasonable strategy for more advantages. It has been shown that LBs, the pathological features of PD, can be found in grafts of foetal VM tissue (100,101). The first reason is that LB pathology is a reaction to inflammation from host brain tissues, possibly mediated by cell stress induced by reactive microglia (102). Second, the spread of SNCA into the graft from the host may contribute to these pathological changes (103,104). However, Barker *et al.* believe that these pathological changes are not likely to limit widespread adoption of cell treatments, as numbers of cells with LB-like pathology in the grafts were small compared to numbers of healthy cells (105–107). Patients can still be functionally stable, more than a decade after such a graft, at a time when accumulation of SNCA had been observed (108,109).

Apart from the potential risk of tumorigenicity or inherent pathogenic features deriving from donor cells, pluripotent stem cell-derived cell populations for therapies also confer a risk for the contamination of transplantation cell populations with residual pluripotent cells (110). In order to resolve this issue, several sorting methods have been developed for enrichment of differentiated neural cell populations and elimination of pluripotent stem cells, using flow cytometric analysis (FACS) or MACS (111). Different combinations of CD markers have been explored to purify heterogeneous pluripotent stem cell-derived neural cell populations. Pruszkowski *et al.* identified a cluster of differentiation (CD) surface antigen code for the neural lineage, based on combinatorial FACS of three distinct populations derived from hESCs: combinatorial CD15/CD24/CD29 marker profiles (112). They found that CD15(+)/CD29(HI)/CD24(LO) surface antigen expression defined NSCs, and this could eliminate tumour formation *in vivo*, resulting in pure neuronal grafts (112). Yuan *et al.* performed an unbiased FACS- and image-based immunophenotyping analysis using 190 antibodies to cell surface markers on pluripotent stem cells (113). From this analysis they isolated a population of NSC that were CD184(+)/CD271(-)/CD44(-)/CD24(+) from neural induction cultures of hESCs and hiPSCs (113). To improve the sorting method, Sundberg *et al.* sorted primate iPSC-derived neural cell population with

NCAM+/CD29^{low} selection (111). They demonstrated that the NCAM+/CD29^{low} selection method enriched the FOXA2/TH and EN1/TH⁺ DA neurons *in vitro* compared to unsorted cell populations from >10% prior to sorting to > 35% after sorting. Importantly, sorting with NCAM+/CD29^{low} selection prior to transplantation eliminated non-neural tumorigenic cells from the grafts and significantly increased the number of TH⁺ cells in the cell grafts compared to unsorted cell populations (111).

Conclusion

In this review, we have summarized a number of scientific and ethical issues in modelling and treatment with iPSCs for PD. iPSCs can be employed as relevant Parkinsonian cell models, for drug screening, studying disease progression and most importantly for treatment of PD by transplantation techniques. Compared with other stem cells, iPSCs stand out for their powerful pluripotency, few ethical issues and less immune rejection, although there are still several issues that need to be solved prior to translation of iPSCs into the clinical setting. First, the exact mechanisms of how transplanted cells restore host brain function and how to connect them with circumjacent brain tissues have not been yet elucidated. Second, tumorigenicity of iPSCs may surpass their therapeutic effects. Ultimately, iPSC, derived from autologous PD patients, may contain pathogenic gene mutations that affect prognosis of transplantation therapy. With improvements in differentiation methodologies and better understanding of pathogenesis of PD through patient-specific iPSCs, iPSC therapy can be a potential alternative for PD treatment combined with traditional drug development platforms and gene therapy.

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Conflict of Interest

There are no actual or potential conflicts of interest.

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