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Human induced pluripotent stem cells in Parkinson's disease: a novel cell source of cell therapy and disease modeling

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Abbreviation of words:

AA: Ascorbic Acid

ALS: Amyotrophic lateral sclerosis

BDNF: Brain derived neurotropic factor

BMP: Bone morphogenetic protein

DNMT: DNA methyltransferase

EB: Embryoid body

EBNA1: Epstein-Barr nuclear antigen-1

ERK: Extracellular signal-regulated kinase

FGF8: Fibroblast growth factor 8

FP: Floor plate

GDNF: Glia cell derived neurotropic factor

GSK-3β: Glycogen synthase kinase-3β

hESCs: Human embryonic stem cells

hiPSCs: Human induced pluripotent stem cells

hTERT: Human telomerase

Klf4: Kruppel-like factor 4

LIF: Leukemia inhibitory factor

Oct4: Octamer-binding transcription factor 4

PD: Parkinson's disease

RSMADs: Receptor-regulated mothers against decapentaplegic+ *Caenorhabditis elegans* protein

SAHA: Suberoylanilide hydroxamic acid

SMA: Spinal muscular atrophies

SHH: Sonic hedgehog

SIP1: SMAD-interacting protein 1

Sox2: SRY (sex determining region Y)-box 2

TGFβ: Transforming growth factor beta

TSA: Trichostatin A

TuJ1: Neuron-specific class III beta-tubulin

VPA: Valproic acid

Wnt: Wingless integration 1

DHA: Docosahexaenoic acid

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

AD: Alzheimer's disease

Abstract

Human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs) are two novel cell sources for studying neurodegenerative diseases. Dopaminergic neurons derived from hiPSCs/hESCs have been implicated to be very useful in Parkinson's disease (PD) research, including cell replacement therapy, disease modeling and drug screening. Recently, great efforts have been made to improve the application of hiPSCs/hESCs in PD research. Considerable advances have been made in recent years, including advanced reprogramming strategies without the use of viruses or using fewer transcriptional factors, optimized methods for generating highly homogeneous neural progenitors with a larger proportion of mature dopaminergic neurons and better survival and integration after transplantation. Here we outline the progress that has been made in these aspects in recent years, particularly during the last year, and also discuss existing issues that need to be addressed.

Key words: Induced pluripotent stem cells, Parkinson's disease, reprogramming, dopaminergic differentiation, neural transplantation, disease modeling

Table of Contents

1 INTRODUCTION 6
1.1 CLINICAL EVIDENCE OF NEURAL TRANSPLANTATION IN PD PATIENTS 6
1.2 HUMAN EMBRYONIC STEM CELLS AND INDUCED PLURIPOTENT STEM CELLS IN CELL REPLACEMENT THERAPY AND DISEASE MECHANISMS 7

2 GENERATIONS OF PLURIPOTENT STEM CELLS – A ROADMAP 8
2.1 THE ORIGINAL PROTOCOL – VIRAL VECTOR APPROACH 8
2.2 USING FEWER, DEFINED TRANSCRIPTIONAL FACTORS 10
2.3 SMALL MOLECULES IMPROVE THE EFFICIENCY FOR REPROGRAMMING 11
2.4 INTEGRATION FREE REPROGRAMMING 13
2.5 XENO-FREE AND FEEDER FREE CULTURING FOR FUTURE CLINICAL APPLICATION 15

3 SUFFICIENT DOPAMINERGIC DIFFERENTIATION: 16
3.1 SUPPLEMENTATION OF COMPOUNDS AND GROWTH FACTORS PROMOTES DOPAMINERGIC DIFFERENTIATION 17
3.2 SIGNALING PATHWAYS INVOLVED IN DOPAMINERGIC CELL DIFFERENTIATION 17
3.2.1 Wnt signaling pathway 17
3.2.2 BMP/SMAD signaling inhibition: 18
3.2.3 SHH pathway and FGF8: 19
3.3 A SYMPHONY OF SIGNALING PATHWAYS 20
3.4 APPLICATION OF FACS SORTING TO INCREASE DOPAMINERGIC NEURON POPULATION AND PURITY 22

4 APPLICATION OF INTRA-CEREBRAL TRANSPLANTATION 24
4.1 SURVIVAL OF TRANSPLANTED CELLS VERSUS PHENOTYPIC STABILITY 24
4.2 PROMOTION OF INTEGRATION OF TRANSPLANTED NEURONS WITH THE HOST BRAIN 24

5 INDUCED PLURIPOTENT STEM CELLS: BEYOND CELL THERAPY 27
5.1 UNIQUE MODELS TO STUDY DISEASE MECHANISMS 27
5.2 DRUG DEVELOPMENT WITH IPSCS 33
5.4 ESTABLISHMENT OF BIOBANK SYSTEM FOR HIPSCS-- A DIRECT COMPARISON BETWEEN AUTOLOGOUS AND ALLOGENEIC TRANSPLANTATION 36
5.5 FUTURE CHALLENGES FOR HIPSC/HESC APPLICATION 37

6 PERSPECTIVE 39

7 REFERENCES 40

1 Introduction

1.1 Clinical evidence of neural transplantation in PD patients

Parkinson's disease (PD) is the most common movement disorder in man. Loss of dopaminergic neurons in the substantia nigra pars compacta is the pathological hallmark of the disease. Therapies for PD have been mainly restricted to the relief of symptoms and there is still no effective and reliable model for studying the mechanisms of the disease.

Since the 1980's, several open-labeled trials have been performed to transplant human fetal dopaminergic neurons into the putamen and/or caudate nucleus as a replacement therapy to restore dopaminergic transmission[1, 2]. Concrete evidence shows that neural transplants survive and become functional and integrated with the host brain neurons[3, 4]. Fluoro-Dopa PET scans demonstrate that grafted neurons can actively take up dopamine, reflecting functional neurons in the transplant; the patients show benefits in general performance, reduced rigidity and increased speed of movement[5, 6]. Post mortem studies of the brain tissues that received neural transplantation demonstrate the long-term survival of dopaminergic neurons, even longer than two decades post-operation (Fig 1). These open-labeled clinical trials show that, when successful, dopaminergic neuron transplantation can be beneficial and promising to PD patients. However, two NIH-sponsored double-blind placebo trials failed to reach the primary outcome, i.e. a significant benefit to the patients[7, 8]. Furthermore, a considerable number of transplanted patients developed clear adverse effects – graft-induced dyskinesia. After intensive debates on clinical trial designs of cell replacement therapy, including patient selection, graft tissue preparation and processing and optimization of surgical procedures, a new clinical trial (TRANSEURO) was established and is currently ongoing cross-continentally in multi-research and clinical centers[9]. Success of this trial will have a major impact on neural transplantation with fetal dopaminergic tissues, and also with human embryonic stem cell (hESC)- and human induced pluripotent stem cell (hiPSC)-derived dopaminergic neurons in future.

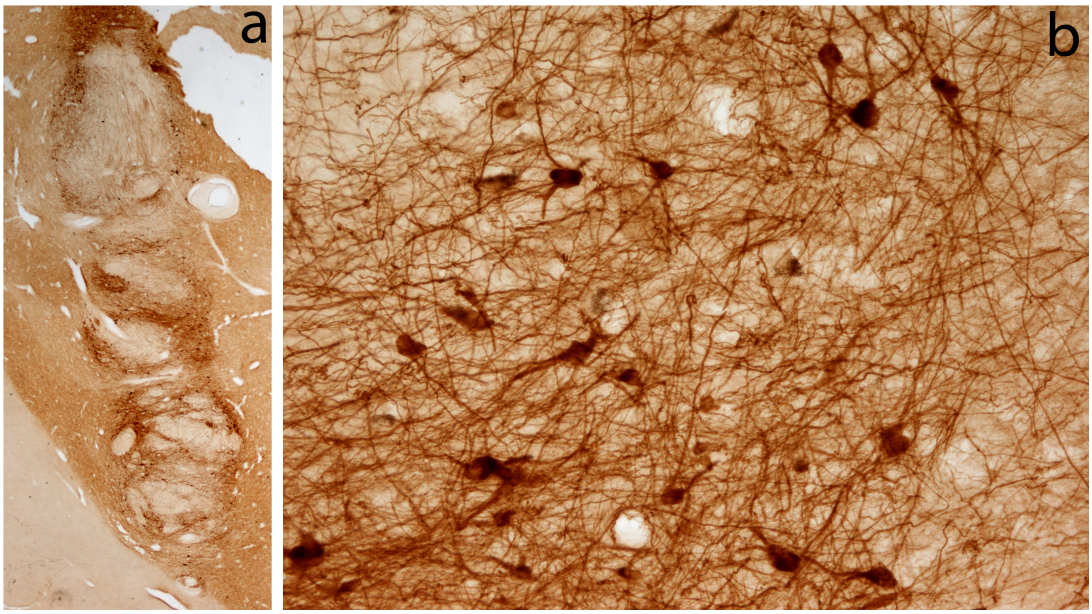


Fig.1. Microscope images, showing long-term survival of dopaminergic neurons 16 years after transplantation into the putamen of a PD patient. The neurons are labeled with an antibody against tyrosine hydroxylase, the key enzyme of dopamine synthesis. The low power image (a) depicts the grafts in the putamen, while the high power image (b) shows that the surviving cells exist in the periphery of the graft with good morphology of dopaminergic neurons and also exhibit extensive dopaminergic fiber networks within and outside the graft.

1.2 Human embryonic stem cells and induced pluripotent stem cells in cell replacement therapy and disease mechanisms

Embryonic stem cells (ESCs) provide hope for regenerative medicine, and have been proposed as a source of donor cells for replacement therapy in PD. ESCs are pluripotent; they have a wide differentiation potential to generate tissues and cells derived from all three embryonic germ layers. Mouse ESCs can be differentiated into dopaminergic neurons[10, 11] with efficient survival rates and can give rise to functional recovery after transplantation into the brains of rodent models of PD[12, 13]. However, it has been difficult and complicated to generate high yields of dopaminergic neurons from hESCs with various differentiation protocols. Furthermore, following intra-cerebral transplantation, the survival of transplants and functional effects have not been satisfactory, even with reports of tumor or teratoma formation[10, 14]. HiPSCs[15] are promising potential cell sources for studying neurodegenerative diseases and may, in the future, be used for cell therapy as well. From the

perspective of the differentiation potential, hiPSCs encounter similar problems to hESCs. The application of hiPSCs/hESCs in PD research has been substantially limited by the lack of effective protocols for differentiation and transplantation. A series of studies have been undertaken to optimize protocols of hiPSC/hESC differentiation and transplantation; although improvements have been made in the last few years, many problems still exist. Recently, a breakthrough of differentiating hESCs *in vitro* broke the deadlock[16, 17]. Here we will discuss advances that have been made in terms of dopaminergic conversion from hiPSCs/hESCs and transplantation studies in recent years and summarize the state-of-the-art development and prospects for effective and safe use of hiPSCs/hESCs for PD therapy in the future. We will keep to the following outline: hiPSCs/hESCs → neural progenitors → mature dopaminergic neurons → transplantation, and specify the progress that has been made in each of these aspects, including advanced reprogramming strategies without the use of viruses or using fewer transcriptional factors[18-21], the optimal methods for generating highly homogeneous neural progenitors and a greater proportion of mature dopaminergic neurons[17, 22-26]. Furthermore, we will focus on the survival, integration and safety issues regarding teratoma/tumor formation after intra-cerebral transplantation. This review will provide a timely highlight of recent advances and a better understanding of the use of hiPSCs/hESCs in cell therapy and in disease mechanism studies of PD for both clinical neurologists and researchers.

2 Generations of pluripotent stem cells – a roadmap

2.1 The original protocol – Viral vector approach

Traditionally, reprogramming of somatic cells to pluripotency has been achieved by two different methods. Firstly, nuclear transfer — transplanting nuclei from differentiated somatic cells into oocytes. Secondly, cell fusion — involving fusion of two or more cells into one, which reveals the fact that silent genes in differentiated cells can be activated by certain regulators[27].

In 2006, Yamanaka and co-workers showed that somatic cells can be reprogrammed into an

embryonic-like state by introducing 4 transcriptional factors, Octamer-binding transcription factor 4 (Oct4), SRY (sex determining region Y)-box 2 (Sox2), C-myc and Kruppel-like factor 4 (Klf4), into embryonic mouse fibroblasts. These reprogrammed cells were designated as induced pluripotent stem cells (iPSCs), which possess characteristics of typical embryonic stem cells[28]. Since then, a lot of effort has been put into improving approaches to generate iPSCs. For example, in addition to the four factors mentioned previously, Thomson and colleagues reprogrammed human fibroblasts with a distinct set of transcription factors comprising Oct4, Sox2, NANOG, and LIN28 (OSNL)[29].

However, most of the methods that have been developed at that time involve the use of viruses, either retrovirus or lentivirus, through which reprogramming genes are integrated into the host genome[30]. The application of viruses and oncogenes into the genome, however, gives rise to safety concerns and limits the potential clinical use of iPSC-derived cells in the future.

Plath and colleagues recently provided further insight into the roles of the four reprogramming factors[31]. They found that ectopic expression of C-myc promotes the most prominent ESC-like expression pattern among the four factors when expressed individually in fibroblasts, and that C-myc functions predominantly prior to the induction of pluripotent regulators during reprogramming. At the final phase of reprogramming, Oct4, Sox2, and Klf4 re-establish an ESC-like association with co-bound target genes and activate other genes of pluripotency. In contrast, partially reprogrammed cells lack similar co-binding activities and may reflect incomplete epigenetic changes and/or the requirement for additional induced factors to cooperatively bind target genes with Oct4, Sox2, and Klf4. However, there is also discussion regarding the role of myc in the use of direct reprogramming. Although addition of c-myc strongly promoted the efficiency of reprogramming, this could increase the risks of tumor formation[32, 33]. C-myc reactivation induces tumor formation due to its transformation activity. Nakagawa *et al* found that another isotype of Myc gene, L-myc, and different mutant forms of C-myc (W136E and dN2) possess a larger tendency to reprogramming and at the same time lower risk of tumor formation compared to C-myc[34].

Increasing evidence shows that generation of iPSCs can be achieved by using fewer, defined factors, applying chemicals or small molecules to improve the efficiency and stability of reprogramming and developing non-integrated strategies to reprogram somatic cells. So, we will discuss the progress that has been made in these aspects below.

2.2 Using fewer, defined transcriptional factors

Originally, Yamanaka and his co-workers used four different factors: Oct4, Sox2, C-myc and Klf4[28]. Researchers first began to optimize the protocol for reprogramming by reducing the number of factors transduced with viruses. One of the main problems with these traditional reprogramming methods is the use of exogenous transcription factors. Viral-dependent transduction of these factors into cells causes permanent integration of transgenes into the human genome. This increases the risk of reactivation of viral genes or even alterations of the human genome later on. Furthermore, two of the four factors, C-myc and Klf4, are oncogenic[33] and two (Klf4 and Oct4) can cause dysplasia[35, 36]. Reducing the number of factors, especially omitting the oncogenic genes used in reprogramming, will hopefully decrease the risk of mutagenesis and also gives hope to the avoidance of using transcription factors in the future. Yamanaka's group made the first breakthrough in 2008; they demonstrated that iPSCs can be transduced by introducing only three factors, eliminating the use of C-myc in both human and mouse fibroblasts, with a longer duration of puromycin selection[37]. Then, Li *et al.* removed Sox2 from the cocktail in 2009[38], using a GSK-3 inhibitor as a replacement for virus-induced factors. Huangfu *et al.* reported the elimination of two oncogenic factors, Klf4 and C-myc[39]. Their work shows that valproic acid (VPA), a histone deacetylase inhibitor, can help to reprogram primary human fibroblasts with only two factors, Oct4 and Sox2. By using a different type of cell, dermal papilla cells rather than fibroblasts, which are a type of skin cell related to hair morphogenesis and regeneration[40], Michael *et al.* generated iPSCs using only 2 factors, Oct4 and Klf4, with a much higher efficiency and a faster process. They continued improving this protocol by using only Oct4 with neural stem cells later [21, 41, 42].

2.3 Small molecules improve the efficiency for reprogramming

In addition to improving reprogramming by reducing the number of transgenes, researchers have found several chemical compounds and small biologically effective molecules that can facilitate the reprogramming procedure.

Reprogramming somatic cells into an embryonic status has been proven possible, however, the main drawback of low efficiency remains. Several compounds and molecules are demonstrated to have an enhanced effect on the reprogramming procedure. 1) Epigenetic reprogramming: SV40 large T antigen (SV40 LT) and the catalytic subunit of the human telomerase (hTERT) have been shown to be able to increase the reprogramming efficiency of human fibroblasts without any genomic integration [43, 44]. The fact that induction of DNA methyltransferase (DNMT) improves reprogramming reveals a role of epigenetic effects to cell reprogramming[45-47]. Addition of the DNA methyltransferase inhibitor AZA increases reprogramming efficiency 12-fold and also increases colony number 4-fold[45]. Both of these findings imply that DNA methylation plays an important role in epigenetic reprogramming[46-48]. 2) microRNA: as an epigenetic modification, manipulating microRNA can enhance the efficiency of reprogramming as well. For example, miR-291-3p, miR-294 and miR-295, the miR 290 cluster, which are specifically present in ESCs, increase the efficiency of reprogramming without the presence of C-myc [49]. miR-302 has a similar effect[50]. 3) p53: knockdown of p53 has also been shown to enhance the generation efficiency of iPSCs from somatic cells[51-55]. Recent studies have revealed interacting effects of microRNAs and p53[54]. miR138 promotes reprogramming by suppressing p53 expression by binding to the 3' untranslated region (UTR) of p53) Histone deacetylase (HDAC) inhibitors: Another very important chemical family, which can enhance reprograming, are the histone deacetylase (HDAC) inhibitors. The most commonly found members are valproic acid (VPA), trichostatin A (TSA), and suberoylanilide hydroxamic acid (SAHA). All three are demonstrated to have enhancing effects on the reprogramming process, either by increasing the efficiency by up to 100-fold or by decreasing the

number of transcriptional factors needed[48, 56]. Besides the chemicals described above, some signaling pathways are believed to participate in the reprogramming process as well. Traditionally, leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP) pathways have been shown to have functions in maintaining the ESC in a pluripotent state. These factors are used in ESC maintenance *in vitro*[57, 58]. Likewise, the inhibition of Extracellular signal-regulated kinase (ERK) and GSK-3 pathways can either maintain the self-renewal state of ESCs or help to induce the reprogramming[58, 59]. Similar effects have been found in the Transforming growth factor beta (TGFβ) and the Wingless integration 1 (Wnt) pathways[60-62]. Here we summarize different factors that have been tried in iPSCs programming (Table 1).

Table 1: Small molecules that improve the efficiency for reprogramming

	Factors	Function	Trans-factors	Cell type	Efficiency and speed	Ref
Epigenetic reprogramming	SV40 LT	Supplementing factors	*Oct4/Sox2/NANOG/ LIN28*	Human fibroblasts	1-2 weeks faster, enhanced efficiency by 23- to 70-fold	43
	hTERT	Transcriptase	OSKM	Human fibroblasts	~3-fold	44
	RG108	DNA methyltransferase inhibitor	OK	Mouse fibroblasts	BIX-01294 + RG108 enhances efficiency ~30 times	63
	BIX-01294	histone methyl- inhibitor	OK	Mouse fibroblast	5 fold	63
	DNMT	Inhibition of DNA methyltransferase	OSKM	Mouse fibroblasts	promotes full reprogramming	45, 56
	BayK8644	L-type calcium channel agonist	OK	Mouse fibroblasts	BIX-01294 + BayK8644 enhances efficiency ~15 times	63
Histone deacetylase (HDAC)	Butyrate	histone deacetylase inhibitor	OSKM	MEF	A maximum 7-fold increase	64
	AZA	DNA methyltransferase inhibitor	OSKM	Mouse fibroblasts	~10-fold increase in efficiency	48
	VPA	histone deacetylase inhibitor	OSKM	Mouse fibroblasts	greater than 100-fold increase in efficiency	48
	TSA	histone deacetylase inhibitor	OSKM	Mouse fibroblasts	~15-fold increase in efficiency	48
Signaling pathways	Wnt3a	Cell signaling molecule	OSK	Mouse fibroblasts	~20-fold increase in efficiency	61
	Sodium butyrate SB431542 PD0325901	Inhibition TGF β /MAPK/ERK Histone deacetylase inhibitor	OSKM	Human fibroblasts	As high as a ~93% reprogramming efficiency	65
	RAR- γ	RA receptors (RARs) or RA agonists	OSKM	Mouse+ Human fibroblasts	4 day induction Efficiency: 80% of reprogramming	**66**
P53 gene and miRNA	p53 siRNA,	Maintain self-renewal state	OSKM	Human fibroblast	~100-fold increase in efficiency	67
	Tranilast	Activation of AhR aryl hydrocarbon receptor (AhR) promoting miR-302 expression	OSK	*Mouse Embryonic Fibroblast (MEF) Cells*	7 -old	68
	miR-290 cluster miR-302d	Substitutes for cMyc	OSK	Mouse fibroblasts	Increasing efficiency from 0.01–0.05% to 0.1–0.3%	50, 62

Others	Depletion of Mbd3	Restrains reactivation of OSKM downstream target genes	OSKM	Mouse+ Human fibroblasts	Deterministic and synchronized iPS cell reprogramming (near 100% efficiency within seven days)	[69]
	Hypoxia	Maintain self-renewal state as an environment factor	Oct3/4, Sox2, Klf4, +/− c-Myc	Mouse+ Human fibroblasts	Mouse: increased 7.4-fold increased 20-fold without myc Human: around 2- to 3-fold	[70]
	RepSox	Replacement of SOX2	OKM	Mouse fibroblasts	Show colonies, Short to one day treatment	[71, 72]

2.4 Integration free reprogramming

One of the safety concerns in using iPSCs in clinical regenerative medicine is the use of viruses. Application of retroviruses and lentiviruses causes genomic integration of the virus gene and transgene, raising the risk of tumorigenicity and also reactivation of the virus gene[73]. To solve this problem, a great deal of effort has been made to find potential alternatives as carriers to deliver the transgene into host cells without genomic integration. In 2008, Hochedlinger *et al.* generated functional iPSCs without the use of integrating viruses by employing adenoviruses either alone or in combination with an inducible transgene [74]. The use of adenovirus makes it possible to avoid host genome integration of the transgenes. Another safer virus for generating non-integration iPSCs is the Sendai virus, which was reported by Hasegawa's lab in 2009[75]. The Sendai virus is a negative-sense single-stranded RNA in the cytoplasm of infected cells, which does not go through a DNA phase or integrate into the host genome. These viruses show highly efficient introduction of foreign genes[76]. Later, Yamanaka's group made additional achievements by using two complementary DNAs (cDNAs) to transduce the four transcriptional factors into mouse fibroblasts[73]. They also used retrovirus and adenovirus, but only to test the constructs of plasmids; the final transfection was performed without any virus. They were able to generate iPSCs without any gene integration through optimization of the transfection protocol. However, drawbacks of the integration-free methods include slower procedures and lower efficiency of reprogramming[73]. In 2009, a study found a solution to this efficiency problem. One single multi-protein expression vector, which comprises the coding sequences of C-myc, Klf4, Oct4 and Sox2, linked with 2A peptides, is used as a transporter. The 2A peptide is a region of the sequence of the foot and mouth disease virus (F2A), which has been used as a multi-protein vector in cell transient reprogramming[77]. In this study,

pCAG2LMKOSimO, which has C-myc, Klf4, Oct4 and Sox2 coding regions linked with 2A peptide sequences driven by a CAG enhancer/promoter, was constructed. The multi-protein expression of this vector resulted in a robust level of C-myc, Klf4, Oct4 and Sox2, which then resulted in highly efficient generation of functional iPSCs, as demonstrated by *in vitro* differentiation assays and formation of adult chimeric mice. This method was then further validated in both mouse and human cells. Most importantly, they managed to remove the transgene once reprogramming was achieved, using a transient Cre transfection[78-80]. A similar method was reported in 2009 for generating a PD-specific iPSC line using loxP-flanked vectors followed by Cre-recombinase-mediated excision [81]. However, while all of these methods are able to eliminate transgenes from host cells, the vector constructs they use still remain in the host cells; these genes remain potential risk factors for mutagenesis in the future. In 2009, Thomson and co-workers reported the first study that enabled the excision of both transgene and vector sequences. Using an oriP/ Epstein-Barr nuclear antigen-1 (EBNA1)–based episomal vector, both transgene and vector gene could be easily removed from the host cells when cultured without drug selection[82].

Another important milestone of cell reprogramming was the use of protein-based transduction. Without the introduction of any transgenes or nucleotide vectors, researchers used proteins derived from ESCs as pluripotency carriers. This system eliminates the potential risks associated with the use of genetic material and/or potentially mutagenic molecules. By fusing the reprogramming proteins to a penetrating protein, it enables the transcriptional proteins to enter the host cells[20, 83]. Shortly after this, Cho *et al.* reported a method of using ESCs–derived extract proteins, transferred by streptolysin O-mediated reversible permeabilization – an already commercially available penetration protein[84]. Unfortunately, the use of protein based reprogramming results in a lower efficiency.

All of the studies above managed to either reduce or eliminate the use of viral vectors from the cell reprogramming procedure, which lowers the concern about host genome integration and later risk of mutagenic changes. Overcoming these obstacles will improve the possibility of using iPSCs in

clinical regenerative medicine. However, the reprogramming protocol still requires further optimization, particularly in improving the relatively low efficiency as compared to viral vector methods[84].

2.5 Xeno-free and feeder free culturing for future clinical application

In addition to the viral introduction during reprogramming, another obstacle that prevents the clinical application of hiPSCs is the use of xeno biotic materials and animal origin feeder cells[85]. The use of feeder cells and xenogeneic materials will result in contamination of animal source pathogens and may trigger immune reactions when applied for transplantation therapy[86]. In 2005, researchers observed animal source immune active forms of NeuG5 in hESCs after the use of animal derived materials during differentiation[86]. Therefore, the use of xeno-free and feeder-free methods is definitely necessary for the clinical application of hiPSCs. Several steps in the reprogramming and proliferation of hiPSCs are involved with animal-based or undefined factors[87]. 1) Cell surface: in order to help the establishment of clones during reprogramming and increase efficiency, generations of hiPSCs often include feeder cells or a matrigel coated cell surface[88]. The most common used feeder cells are mouse embryonic fibroblasts (MEF). Immortalized human skin fibroblasts have been shown to support the generation and expansion of hiPSCs, which is a promising source of feeder cells in the future[89]. Extracellular matrix such as matrigel and laminin are also suggested to be useful material for surface of reprogramming. However, there is evidence showing the extracellular matrix may decrease the pluripotency of induced hiPSCs and increase the risk of mutation[90]. The components of this matrix often include animal derived materials, which will raise the safety issue later on. 2) Culture media: fetal bovine serum and modified version of serum replacement have been widely used in the reprogramming and expansion process. Other factors such as trypsin and dispase for dissociating cells are also used in the generation of hiPSCs. Xeno-free media contain human plasma based serum replacement and also define all the compartments in the medium. This is widely used to develop xeno-free generation of hiPSCs. Rajala *et al* used xeno-free medium (RegES)

composed of a knockout-Dulbecco's modified Eagle's medium. They generate hiPSCs, which maintain pluripotency through culture and express surface markers. They used human fibroblasts to support the reprogramming process. hiPSCs generated this way can survive and maintain morphology and pluripotency up to 20 passages[91]. Rodriguez-piza *et al* used human fibroblasts as both the cell source for reprogramming and as feeder cells. Through culturing in human plasma based xeno-free medium, they generate hiPSCs that express pluripotency markers and can be functionally differentiated[87]. Recently Chou *et al* succeeded in using a human recombinant vitronectine protein substrate surface to support the reprogramming of mononuclear blood cells. Combined with xeno-free culture medium, they generated clinical compliant hiPSCs in a high efficiency[92].

3 Sufficient dopaminergic differentiation:

Degeneration of dopaminergic neurons in the substantia nigra is the pathological hallmark of PD. Using iPSCs for cell replacement therapy has been considered as a promising approach for the treatment of PD. However, the realization of this potential has been largely hindered by several problems, especially the low efficiency of generating a large quantity and well-defined population of dopaminergic neurons from iPSCs[93]. Previous discussions regarding differentiation efficiency have focused on the use of non-human feeder cells or genetic modification of stem cells to obtain a high yield of dopaminergic neurons. The use of non-human feeder cells such as murine stromal cells-PA6 and MS5 cells[14, 94, 95], can increase differentiation efficiency to a certain extent but also introduces cells of non-human origin into the culture, which will have a negative impact in clinical applications. Modification of the stem cell genome by introducing genes that promote dopaminergic neuron differentiation will give rise to a high yield of TH positive cells. However, this method introduces exogenous transgene and also vector DNA into cells, which may cause unpredictable mutagenic risks in prolonged passages. Hence, it will not be the preferred method in cell replacement therapy [96-98].

3.1 Supplementation of compounds and growth factors promotes dopaminergic differentiation

Various growth factors and soluble compounds or their combinations have been used to improve neural induction and dopaminergic differentiation. Several different factors have been used to promote these processes, such as Fibroblast growth factor 8 (FGF8), FGF20, Brain derived neurotropic factor (BDNF), ascorbic acid (AA), Glia cell derived neurotropic factor (GDNF) and Sonic Hedgehog (SHH)[14, 94, 99-103]. From the aspect of developmental biology of the brain, in order to generate specific neurons, such as dopaminergic neurons, reminiscent of the neurons *in vivo*, one must mimic the temporal neuronal differentiations with close correlative administration of specific growth/trophic factors in defined dose-dependent manners. However, in many previously published studies, the use of these growth factors has not been based on a good understanding of relative signaling pathways. What's more, these methods often require co-culturing with stromal cells and have a long *in vitro* culture period[93]. There is still a room for improvement in using these factors for neural induction and dopaminergic differentiation.

In summary, the critical issues in using multiple factors to improve the differentiation efficiency can be simplified as 1) the understanding of how different signaling pathways function and interact in dopaminergic differentiation, and 2) how to achieve a relatively clean, feeder-free and more efficient method of differentiation to facilitate future clinical applications.

3.2 Signaling pathways involved in dopaminergic cell differentiation

3.2.1 Wnt signaling pathway

Wnts are 350–500 amino acids long secreted proteins, which are involved in diverse cellular functions, such as cell self-renewal, cell proliferation and differentiation, and embryonic patterning[104, 105]. Abnormality of Wnt signaling can result in diseases such as tumor genesis[104].

Wnts have 19 different isotypes involved in both canonical and non-canonical pathways[106]. There is widespread Wnt expression in the central nervous system, which implies the important function of Wnts in neural regulation. Evidence has pointed out that WNTs play a critical role in both early and

late stages of neural development, including neural induction, progenitor proliferation[107, 108], anterior and posterior patterning[109, 110], neural differentiation, axon guidance, and neural integration and migration[107, 108, 111-113] Most importantly, Wnts are crucial for midbrain dopaminergic development. It has been shown that the Wnt family is expressed in developing midbrain, which suggests the role of Wnts in midbrain patterning[114]. Wnts are expressed in dopaminergic precursor cells. Wnt-1, Wnt-3a and Wnt-5a can all increase the number of TH positive cells, although they may act through different mechanisms. Wnt-3a promotes the proliferation of Nurr1-positive (orphan nuclear receptor-related factor 1) precursor cells while Wnt-1 predominantly increases the TH+ cell percentage, and Wnt-5a increases the mRNA levels of some mature dopaminergic markers such as TH and c-ret, thus increasing the proliferation of both total neuron and TH+ neuron populations. This implies that it increases the neuron number without compromising the dopaminergic proportion[115]. Several studies have reported examples of Wnts facilitating midbrain differentiation. Indirubin-3-monoxime and kenpaullone, two chemical inhibitors of glycogen synthase kinase Glycogen synthase kinase-3β (GSK-3β), increase neuronal differentiation in ventral mesencephalon precursor cultures. GSK-3β is a main component of the canonical signaling in Wnt. The inhibition of GSK-3β can stabilize β-catenin protein. It has been found that these two proteins increase both the number and percentage of TH+ cells[116]. Studies by Arenas's group found that purified Wnt5a and Wnt3a can improve the differentiation into dopaminergic neurons by activating both canonical and non-canonical pathways[117]. Researchers have also found that secreted Frizzled-related proteins (sFRPs), a family of proteins that modulate Wnt signaling, can regulate development of dopaminergic neurons by activating the Wnt/planar-cell-polarity/Rac1 pathway in dopaminergic cells[118].

3.2.2 BMP/SMAD signaling inhibition:

Different evidence shows the critical role of SMAD signaling inhibition in dopaminergic neuronal induction. Noggin-cDNA containing a single reading frame encoding a 26kD protein, which inhibits BMP, has a neural induction effect in *Xenopus* embryos[119] and possesses a similar function in

mammals.[120]. Likewise, other BMP inhibitors, chodrin and Follistain[121, 122], have similar functions. Noggin has also been tested to promote dopaminergic differentiation. In hESC differentiation, it exhibits a better neuro-ectoderm induction and higher yield of dopaminergic neurons in culture[123, 124]. Furthermore, Noggin has an effect of maintaining long-term neural precursor survival and helps form tumor free integration after transplanting into adult neostriatum[125]. In addition to Noggin, dorsomorphin and LDN-193189 are also wellknown BMP inhibitors that are used for dopaminergic differentiation[24].

LDN or Noggin inhibits the BMPs while SB431542 has recently been found to dorsalise the neural development by inhibiting TGFβ. It prevents the production of Receptor-regulated mothers against decapentaplegic+ Caenorhabditis elegans protein (RSMADs) so that the yield of SMAD4 decreases and the BMP/TGFβ signaling pathway is down-regulated[126]. The combination of LDN and SB431542, and also Noggin and SB431542, has achieved great effects in deriving dopaminergic neurons from hiPSCs/hESCs.[17, 23, 24]

3.2.3 SHH pathway and FGF8:

Sonic hedgehog (SHH) and fibroblast growth factor (FGF8) are traditional factors used in dopaminergic differentiation. SHH is an important protein in the hedgehog pathway and has a function in embryonic development including the patterning of the midline of the brain. It tends to have a polarization function with the help of fibroblast growth factors[127, 128]. FGF8 is a very important member of the FGF family, which is involved in several cellular activities. FGF8 controls biological processes including embryonic brain development and the patterning of the midbrain. It is believed that SHH is expressed along the ventral neural tube and FGF8 is produced at the mid/hindbrain boundary. The interaction of these two signals creates the growing balance for dopaminergic neurons in the midbrain[129].

Additional factors and signaling pathways to this classical combination of SHH and FGF8 have been discovered to regulate dopaminergic differentiation. Rosenthal *et al.* first discovered that SHH

cooperating with FGF8 can induce dopaminergic neurons at ectopic locations with a rat embryo, while SHH alone can only induce dopaminergic neurons at the dorsal ventral axis[129]. They also found that supplementation of FGF4 into the mixture of SHH and FGF8 induces 5-HT neurons instead of dopaminergic neurons[129]. These two factors combined with Nurr1 overexpression have a further enhancing effect on differentiating neural stem cells into dopaminergic neurons. In this study, the genetic modification of Nurr1 overexpression increased the response of neural stem cells toward SHH and FGF8 stimulation[130]. This also implies an interaction of SHH and FGF8 with other factors or signaling pathways. The creative part of this article is the detection of the specific time for SHH and FGF8 to give rise to functional TH+ neurons. Since most of the activities are temporary during human embryonic development, it makes sense to apply inducing factors only during a period of defined time rather than throughout the whole process[131]. These supplemented differentiation protocols mentioned here may have focused on different new compounds or factors such as new feeders[132], retinoic acid[22] or SMAD and Wnt signals[17, 23], but most of them still require the addition of SHH and FGF8.

3.3 A symphony of signaling pathways

Due to the complexity of the neuronal structure and networks, neuronal differentiation is a process that requires coordinated actions of different factors and signaling pathways. This has been proven to be the case for dopaminergic differentiation of hESCs/hiPSCs. In the process of midbrain patterning, multiple interactions between different pathways have given hope for differentiation with high efficiency.

The floor plate is an essential ventral midline-organizing center that produces the morphogen SHH, which also functions in the production of midbrain dopaminergic neurons at later stages. Research has shown that interaction of canonical Wnt/β-catenin signaling and SHH may orchestrate floor plate neurogenesis and then decide the future midbrain patterning[133, 134]. Another coordination is between SMAD and Wnt on dopaminergic differentiation. SMAD signaling inhibition acts as an upstream

signal of Wnt signaling through SMAD-interacting protein 1 (SIP1)[135]. Recent studies have greatly improved the understanding of the above signaling pathways to generate a high yield of dopaminergic neurons *in vitro*. Studer's group reported a dopaminergic differentiation protocol, which has a very high efficiency even at the early stage of differentiation[23]. They combine SMAD inhibition together with SHH and FGF8 supplementation. During the first 11 days, the cells are cultured with SMAD inhibitors - Noggin and SB - added to the medium for neural induction. Almost 80% of cells are converted into neural ectoderm cells expressing PAX6. SHH and FGF8 function as midbrain neural patterning factors. With additional factors, such as GDNF and BDNF, they can improve dopaminergic differentiation. By day 19, TH positive cells were found in culture with a 50% proportion[23]. Later, the same group raised a concept about floor plate patterning (FP). They reported a method to induce floor plate neural progenitors to generate region specific neural progenitors by adding Wnt1[16]. Their protocol was further optimized in a later study[24]. With the combination of SMAD inhibition, SHH induction and, particularly CHIR, a Wnt signaling activator, they generated the floor plate midbrain neural progenitors by day 11 of differentiation with an efficiency of around 90 percent in total cell populations. On day 25, a relatively large portion (around 20%) of cells expressing TH were positive for FOXA2 (a floor plate cell marker). By day 50, the stage of dopaminergic maturation, they found 70% of TH+ cells in the whole cell population. With similar approaches, two independent studies further verified the above findings and protocols[17, 136]. The major modifications were refined by doses of CHIR, a GSK-3β inhibitor, which promotes the Wnt pathway. They performed a region specific investigation and found that just by changing the concentration of CHIR they could generate neurons specific for the whole neural tube. With a narrow range of 0.7-0.8 μM of CHIR, midbrain dopaminergic precursors were produced with a relatively pure population. Due to this purity, they could transplant the cells at a very early stage on day10, which is a major advantage. This protocol used an Embryoid body (EB) culture format for neural induction, without the use of Matrigel or other non-human matrix. It could have a promising future

for clinical use as well[17, 136].

All of these results show us a new coming era of generating dopaminergic neurons with a better understanding of the neural development process. In a less time-consuming, but also accurate way, researchers are on their way to generating a larger proportion and purer population of TH+ neurons, which may one day be used in cell replacement therapy.

Here we created a figure summarizing how the three signaling pathways co-operate with each other (Figure 2). We also compiled a table of recent publications on dopaminergic differentiation (Table 2).

Figure 2: Signaling pathways involved in dopaminergic differentiation

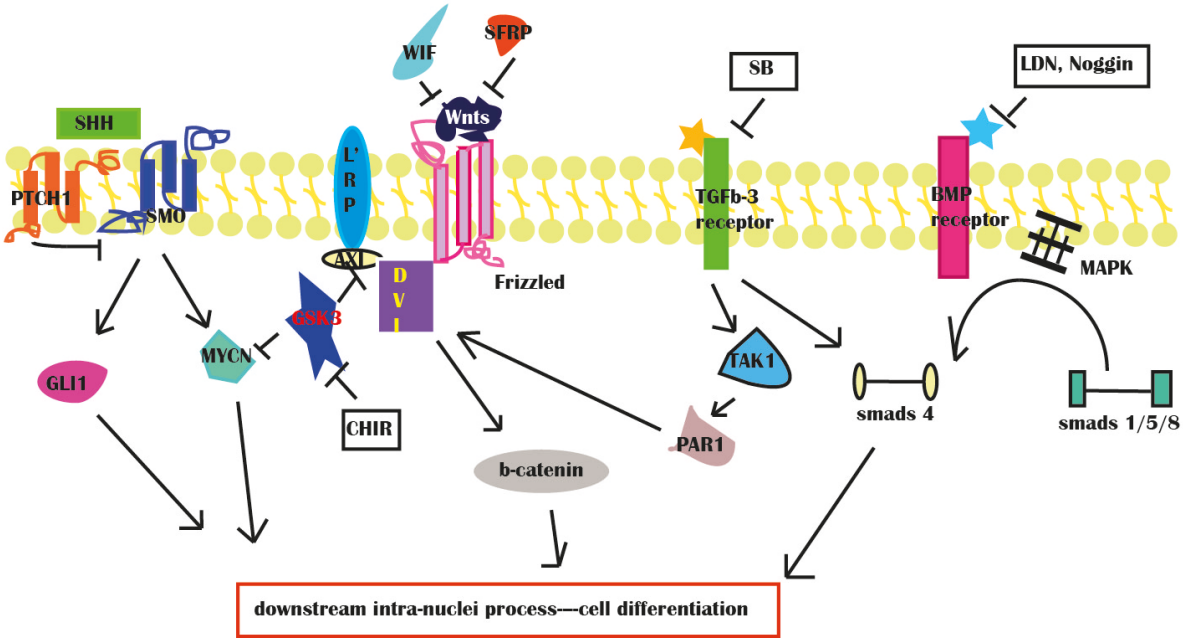


Fig.2. Depiction of three different pathways involved in dopaminergic differentiation and their interaction with each other. The far left of the figure depicts the SHH pathway, the middle shows the Wnt pathway, and the right illustrates the SMAD pathway. In the neural development process, the SMAD pathway is inhibited. Commonly used inhibitors include LDN, SB and Noggin, as shown in the figure. Wnt pathway and SHH pathway need to be promoted in order to facilitate dopaminergic differentiation. Functional receptors and agonists or antagonists are shown here.

3.4 Application of FACS sorting to enrich dopaminergic neuron population

Many differentiation methods result in a mixed population of neuronal and non-neuronal cells. Dopaminergic cells are in mixed culture with glia, neural progenitors, and other neuronal cell types. This may cause risk of tumor formation or overgrowth, especially with neural progenitor cells. Cell

selection can be used to purify certain neuronal phenotype with certain specific cell surface markers[137]. Yosif *et al* use BACs to establish reporter lines, which will turn green at certain stage of dopaminergic differentiation. They differentiate and purify dopaminergic cells effectively for transplantation. With nearly pure population of cell culture, animals receiving transplantation exhibit clear behavior recovery without any tumor formation. Most interestingly, they create reporter lines driven by promoters essential at different stages of dopaminergic development and successfully identify the Nurr1-positive stage as the most appropriate transplantable stage, which resulted in the best survival *in vivo*[137]. Hedllud *et al* also use similar sorting strategies in mouse ESCs[138]. Other studies accent the importance of cell selection in the process of neuronal differentiation. Pruszak *et al* have developed methods of identifying the neuronal population apart from non-differentiated cells by labeling neurons with synapsin I–GFP and immature embryonic cell types, such as SSEA. In this way, they purify differentiated neuronal population from cells maintaining pluripotency, which is essential when it comes to decreasing the risk of tumor formation in transplantation therapy[139].

Table2: Overview of dopaminergic differentiation

Cell lines	Culturing condition	Genetic modification	Factors used	TH+ generation	Ref
hESCs (BG01)	Grown with PA6 mouse stromal cells	---	---	TH+ cells in 87% colonies	140
mESCs	Grown with PA6 mouse stromal cells	Nurr1 overexpression	SHH, FGF-8, AA	56% of total cells 90% of TuJ1+ cells	98
hESCs (H1, H9, HES-9)	Grown with MS5 mouse stromal cells	---	SHH, FGF-8, BDNF, GDNF, TGF-b3, cAMP, AA	19%–39% of total cells 64% TuJ1+ cells	95
mESCs	Grown as EBs	---	bFGF, SHH, FGF-8, AA	5% of total cells (7% of TuJ1+ cells)	99
mESCs	Grown as EBs	Pitx3 or Nurr1 Overexpression	bFGF, FGF8, SHH	25% of TuJ1+ cells	141
HSF-6	Grown with MS5 Mouse stromal cells	Modify with SHH and Bcl-XL gene	SHH, FGF8, Ascorbic acid	13.0 ± 0.6% on D6	142, 143
hESCs	Grown as EBs with primary astrocytes	---	bFGF, SHH, FGF-8	40% of total cells 67% of TuJ1+ cells	10
hESCs hiPSCs 2C6 SeV6	Matrigel, laminin and FN	---	bFGF, SHH, FGF8, LDN SB, CHIR, BDNF, GDNF, AA, cAMP, DAPT	70% of total cells on D50	23, 24
hESCs H9,SA121	EB, laminin and FN	---	bFGF, LDN, SB, SHH, FGF8 and CHIR. BDNF, GDNF, AA, cAMP DAPT	50% of total cells on D42	17
hESCs hiPSCs 4 virus-lines 2 protein-lines	MS5 stromal cells overexpress SHH	---	SHH, FGF8, BDNF, GDNF, AA, cAMP	35-45% of Tuj1+	20

4 Application of Intra-cerebral transplantation

4.1 Survival of transplanted cells versus phenotypic stability

Together with advanced differentiation protocols, the result of research into transplantation has been greatly improved. Dopaminergic neurons, or dopaminergic neural progenitors, can be transplanted at a very early stage (for example, day 10[17]) with an improved survival rate and lower tumor formation. Also, the grafts after transplantation show promising integration with the host brain[99, 144]. Below, we summarize the cell lines, differentiation methods, TH+ cell yields, transplantation time and the survival, integration and tumor formation of grafts from recent studies (Table 3).

4.2 Promotion of integration of transplanted neurons with the host brain

The aim of transplantation is to study defined conditions for cell replacement therapy. For dopaminergic neurons to function properly *in vivo*, one of the key requests that must be met is for the dopaminergic neurons to function properly. This involves two main criteria: 1) TH+ neurons themselves must be mature enough. There should be no sign of tumor formation or any proliferating cells, as we discussed above. Also, the grafted neurons need to be functionally mature with regulated release of dopamine and also electrophysiological characteristics of midbrain dopaminergic neurons[143]. 2) Grafted TH+ neurons must establish local circuits both within the grafts and particularly with host neurons[145-147].

Recent studies regarding cell transplantation mentioned above have done a great job in this aspect[17, 24, 148]. Most of the research discussed here has resulted in good integration between the host brain and grafts. TH fiber outgrowth is often the first sign of innervation commencing, followed by extending into other parts of the brain[146, 147, 149]. However, none of the transplantation studies here have looked into the detailed synapse formation or microscopic connections between individual neurons, which from our point of view could be an area for improvement in the future.

In summary, advances that have been made in the field of cell transplantation therapy for PD are mainly: 1) shorter maintenance time *in vitro*. Transplantation stage can be brought forward to as early as day 10 of differentiation, without compromising the survival of grafts nor increasing the risk of tumor formation[17]. 2) Integration and migration after transplantation are mostly seen in recent cases, with good TH fiber growth and innervation with the host brain[146, 149]. 3) Lower tumor formation is detected, even after long time (up to 4 months) *in vivo*. However, in two recent studies, neural progenitor marker-positive cells or even Ki67 expressing cells are still detected in grafts after one year[146, 147]. This implies that a proportion of grafted cells may still be capable of proliferating, and suggests a potential risk of tumor formation if the animals receiving grafts live for an extended period of time.

Table 3: cell transplantation in recent studies

Cell lines	Differentiation	TH %	Transplantation time	Survival and Integration	Behavior and tumor formation	Ref
O9	SHH/FGF8/Ascorbic acid	6% in Tuj1+ on day23	Day 21	TH fibers into host striatum	Behavior Recovery 4 in 5 2 out of 5 have tumor formation	[99, 144]
HSF-6	1, First into Neural progenitors. 2, BDNF, GDNF, cAMP. 3, MS5 overexpress SHH and Acl-XL. 4, Modify with SHH and Bcl-XL gene;	13.0 % on day 6 after NP	Day 7 to day 15 after NP induction	1,262 ± 207 cells/graft In 483,814 ± 128,923 cells transplanted	Up to 50% of behavior recovery Neuronal proliferation marker continues positive but no morphology of tumor	[142, 143]
H9, H1 hiPSCs 2C6, SeV6	SMAD inhibition, /SHH+FGF8/ WNT activation	On day 50, around 70% of total cells	Day 25 of differentiation	15,000 TH cells in 253105 cells grafted. Massive TH fibers extend to host striatum	A complete rescue of amphetamine induced rotation. < 1% of Ki67+ cells	[23, 24]
H9, SA121	SMAD inhibition, SHH+FGF8 and WNT activation EB, laminin and FN	On day 12, 81% of cells expressing FOXA2 and LMX1A. On day 42, 70% of TH+ cells in total cells	Day 10	After 18 weeks, 54.2% of cells in grafts were TH + Interaction with host striatum	A significant recovery of behavior test. Only a few NESTIN-expressing cells and cell proliferation is minimal	[17]
MR31 MMW2	NSC induction FGF8, SHH and BDNF for the TH differentiation	Approximately 30% of total cells are TH+	Day 20 after NSCs induction	2,106 6 313 TH-positive cells/mm3	Significant rotational improvement at 12 weeks Resembling teratoma-like tissues	[150]
K1, S1, FF17-5, and FF21-26 PD-iPS cells	Stromal cell MS5 SHH/FGF8/AA/BDNF/GDNF/Noggin	Among the Tuj1-positive (30–50% of the total cells) 64–79% of the cells expressed TH	Day 42 of in vitro differentiation	4 weeks, 122 ± 24 TH+ neurons per mm3 16 weeks, 4,890 ± 640 TH+ neurons per graft TH fibers within the grafts and to the host brain	No signs of tumor formation, 0.09% were positive for the proliferative marker Ki-67 All animals show behavior recovery	[95, 145]
WA09 hiPSC lines: 2135 and 1815 Non-human PiPSC lines	Matrigel LDN/SB/SAG/purmorphamine/retinoic acid /SHH/FGF8a/wnt1/noggin Supplemented CHIR	Day 30 FOXA2 >50%–70%, TH >10%–20%, this ratio Increases after sorting	Day30 of differentiation through sorting or without sorting	12359± 2015 TH+ cells per mm^3 unsorted grafts vs. 28,621 ± 6,340 TH+ cells per mm^3 sorted grafts TH+ fiber outgrowth >31,000 TH+ fibers per mm^3	Significant recovery in 16 weeks. 16 weeks later, endothelium cell-type in unsorted overgrowth was detected in the 6-OHDA rat striatum	[147]
4 lenti lines 2 retro lines 2 protein based lines HSF6 and H9	MS5 stromal cells overexpress SHH for NPC induction Supplemented with SHH/FGF8/BDNF/GDN/AA/cAMP	35-45% of Tuj1+ (60-70% of total cells) cells are TH+	Day 4-8 during NPC or terminal 5 days of differentiation around day 7-11	**Moderate concentration NPCs:** TH+ cells (26,882 ± 9,089 cells/graft)	**Moderate concentration NPCs**: Moderate but significant level of functional recovery was observed No tumor growth	[20, 146]

5 Induced pluripotent stem cells: beyond cell therapy

5.1 Unique models to study disease mechanisms

A number of cell and animal models have been used in PD research. Among these models, more and more genetically modified models, either knockout or transgenic, expressing or silencing different PD related genes, have been generated. Use of these models substantially deepens our understanding of PD pathogenesis and helps us to search for disease modifiers and novel targets for possible therapeutic intervention. These models have played important roles in the progress of PD research. However, they all have drawbacks that limit their further applications. Animal models involve large quantities of work and have differences from the real human situation since the most commonly used animals are rodents. Monkeys and other non-human primates have also been used, but only in a very limited number of studies[151, 152]. The cell lines used are often immortal cell lines, which are created either through gene modification or from tumor cell lines so that they can be maintained for extended generations *in vitro*. These have all been very useful as tools for neural toxicity and proteomic studies, but each is limited in its relevance to PD in one way or another. Their non-neuronal nature and variable genetic backgrounds have restricted them as reliable disease models.

Compared to the previous examples, iPSC derived from PD patients are thus promising models for PD study in many aspects.

1. Accurate modeling of disease

iPSCs are often generated from patient somatic cells with exactly the same genetic background as the patient. This makes it possible to study early onset molecular changes during disease progress.

Genetics in PD

PD is attributed to multiple factors. Although over 90% of PD cases are sporadic[153], with or without the possibility of genetic susceptibility, genetic influence still plays an important role in PD etiology. Eleven mutations out of 16 disease loci have thus far been proven to lead to PD pathology, including LRRK2, PINK1 and SNCA.

α-Synuclein is the key protein in PD pathological changes; aggregation of this protein causes the formation of Lewy bodies. The α-synuclein gene (SNCA) was the first dominant gene found to cause inherited familial PD. SNCA mutations include p-H50Q[154], Ala30Pro[155], Ala53Thr[156], gene triplication[157] and E46K[158, 159]. Each of these is a very well defined mutation related to PD. SNCA gene triplication is for now the most studied mutation in iPSCs. This mutation has been proven to have a perturbing effect on iPSCs derived neurons. After successfully differentiated into dopamine neurons, cells bearing the SNCA triplication exhibit disease phenotypes such as accumulation of α-synuclein. They are also more sensitive to oxidative stress and more vulnerable to peroxide challenge[157]. SNCA triplication iPSCs carry double the amount of the synuclein gene as compared to control lines. *In vitro* culture environment of these cells also contains double the amount of α-synuclein. This therefore facilitates the onset of early PD and makes these cells a great tool to study protein pathogenesis in PD[160]. Gene correction has been thought to be one possibility for novel therapy in PD. In 2011, Soldner *et al* shows the correction of point mutation A53T and E46K in synuclein gene using a combination of Zinc finger and iPSC[156]. This provides a new direction of therapeutic study for PD.

LRRK2[161] mutations that are related to PD include R1441C and G2019S. G2019S is the most studied LRRK2 mutation in PD. Research has shown that hiPSC-derived dopamine neurons bearing the LRRK2 G2019S mutation suffer greater oxidative stress and show an increasing level of α-synuclein accumulation. When exposed to oxidative stress reagents, such as 6-hydroxydopamine, LRRK2 mutant hiPSC-derived neurons are more vulnerable to caspase-3 challenge and also cell death[162]. LRRK2 G2019S mutation has also been shown to be involved in autophagy pathology in PD progress. LRRK2 mutant iPSC-derived neurons show impaired synaptic and neuritic morphology compared to control iPSC-derived neuronal cells. These diseased cells also exhibit an increased number of autophagy vacuoles, which occurs while the autophagosome clearance level is lowered in those cells. Interestingly, in this research, iPSCs generated from sporadic PD patient tissue also have

the same pathological changes when compared with control lines[163]. A recent study revealed a correlation between the LRRK2 mutation and several PD related transcriptional factors including CPNE8, MAP7, etc. The G2019S mutation of LRRK2 results in dysregulation of these factors, which then contributes to neuronal degeneration. In this study, they also performed gene correction therapy that eliminated the LRRK2 mutation, which rescued some of the expressions and alterations in the diseased neurons. This can be essentially meaningful because it indicates a new promising therapeutic method[164].

PINK1 is a gene encoding mitochondrial kinase, which is related to mitochondrial physiological degradation. The mutation of PINK1 in familial PD causes an impairment of mitochondrion function, which leads to further pathological changes. Knockdown cell models are very commonly used for mechanistic studies of PINK1 mutation in PD. iPSCs with PINK1 mutation provide a more direct view of endogenous PINK1 function. PINK1 mutated iPSCs can be differentiated to dopaminergic neurons, which later exhibit a mitochondrial deficit of recruiting Parkin and an increasing number of impaired mitochondria. They also exhibit a higher vulnerability against ROS stress and dysfunction in mitochondrial respiration. This can be corrected by the presence of exogenous, normal PINK1. Study of PINK1 in iPSCs accents the role of mitochondrial dysfunction in PD pathology. Therefore, this is a successful example of using iPSCs as a genetic model for mechanic study of PINK1 mutation[165, 166].

The advantage of iPSCs as a model here is in giving rise to patient-specific cells. iPSCs make it possible to generate pluripotent cells from any individual in the context of the patient's own particular genetic identity, not only in the case of genetic mutations but also for sporadic forms of the disease[167, 168]. Until now, a number of iPSC lines from PD patients have been generated in published reports, as summarized in Table 4.

Table 4: PD specific iPSC lines

Year	Gene	Mutation	Differentiation	Reference
2010	SNCA	Triplication	Differentiate into dopaminergic neurons using protocol [23]	[160]
2011		A53T	Differentiate into dopaminergic neurons using protocol[95]	[156]
2011		Triplication	Differentiate into dopaminergic neurons using protocol[157]	[157]
2012		Triplication	Differentiate into dopaminergic neurons using protocol[169, 170]	[169]
2014		Triplication	Differentiate into dopaminergic neurons using protocol[24]	[170]
2011	LRRK2	G2019S	Differentiate into dopaminergic neurons using protocol [23]	[162]
2013		G2019S	Differentiate into dopaminergic neurons using protocol[23]	[164]
2014		R1441C, G2019S	Differentiate into dopaminergic neurons using protocol[166, 171]	[172]
2011	PINK	(c.1366C>T; p.Q456X) or (c.509T>G; p.V170G)	Differentiate into dopaminergic neurons using protocol [23]	[165]
2012		Q456X	Differentiate into dopaminergic neurons using protocol[22]	[166]
2012	PARK2	Deletion in exon 3, 5 or 6	Differentiate into dopaminergic neurons using protocol [23]	[173]

Beyond dopaminergic neurons

In addition to dopamine cells, other neuronal or glial cell types are also involved in PD pathogenesis. Astrocytes constitute the main population of glial cells participating in metabolic processes and regulation of ion concentration in the brain[174]. They also play an important role in synaptic crosstalk and the neural repair process[175]. Astrocytes have been recognized to be greatly involved in PD onset and progression. MPTP metabolism, which is directly connected to sporadic PD onset, is processed in astrocytes[176]. NF-E2-related factor (Nrf2) expression in astrocytes has been proven to prevent the neurotoxic effect induced by MPTP[177]. Astrocyte immune reaction plays a crucial neuroprotection role in PD[178, 179]. Astrocytes also directly correlate with PD onset through several PD related genes. The DJ-1 gene encodes a highly conservative protein. The elimination of this gene gives rise to

autosomal Parkinsonism. DJ-1 is commonly known to have a protecting function against oxidative stress in neurons. Thus, it plays an essential role in preventing the process of PD[180]. In 2009, Mullet *et al* investigated the function of astrocytes in PD onset and progression using a DJ-1 knockdown model. They used neuron-astrocyte contact and non-contact co-cultures to show that DJ-1 knockdown in astrocytes impaired their neuroprotective capacity and supporting property for neurons. These findings support the view that astrocytic dysfunction, in addition to neuronal dysfunction, may contribute to the progression of a variety of neurodegenerative disorders[181-183]. In fact, we should consider astrocytes and other non-neuronal cells as possible contributors to the early PD pathogenic mechanisms. Another important point might be that when considering PD treatment, we may need to consider things beyond dopamine neurons, for example whether glial cells are acting as supportive environment or not. Dopamine related therapy alone might be just part of the issue. Likewise, when we are trying to understand PD mechanisms or choosing PD models, we should also explore other cell types in the surrounding environment.

Another directly related astroglial pathology is associated with α-synuclein. In PD, inflammation plays an important role in neuronal degeneration and worsening of the disease[184]. The triggering of the astrocyte inflammation response accounts for a large portion of this. There has been evidence showing that there is direct neuron to astrocyte transfer of α-synuclein through astroglial endocytosis, which results in the accumulation of α-synuclein in astroglia[185]. This will cause inflammatory responses from astrocytes, which later contributes to loss of neurons[186].

In conclusion, when studying PD pathogenesis or therapies, it is important for us to have a view beyond dopamine neurons. Instead, more of the environmental factors, especially glial cells surrounding and supporting dopaminergic neurons should be taken account. This has been a developing application of hiPSCs. Recently, a few studies have examined methods of converting hiPSCs to astrocytes, as a novel model to study astroglial influences on dopamine neurons. In 2011, Krencik *et al* developed a chemically defined method, which generated almost pure population of

premature astrocytes[187]. These precursors show robust similarity to primary astrocytes, including glutamate responses, calcium wave prolongation and also synaptogenesis. These progenitors, after transplantation into rodent brain, survive and mature into astrocytes with association with blood vessels. Following this study, additional reports showed efficient methods for generating astrocytes from hiPSCs *in vitro*[188-190].

In summary, the multi-potential property of hiPSCs gives the possibility of studying cell types other than just dopaminergic neurons in the onset and development of PD. It is essentially important to consider the supporting cell populations when doing mechanistic studies and cell therapy as well. HiPSCs can give rise to neurons and astrocytes with the exact same genetic background. Through co-culture or transplantation studies, not only dopaminergic neurons, but also their environment will be taken into account. This gives us a more comprehensive modeling of the disease.

2. Self-renewal ability and pluripotent characteristics provide an unlimited cell source

Another outstanding advantage of iPSCs is their self-renewability and potential to give rise to almost all somatic cell types of the human body, including cardiomyocytes, neurons, glial cells, etc. iPSCs act like an unlimited source for generating disease-related specific cell types. In researches on neurodegenerative diseases, several disease specific cells have already been differentiated from iPSCs and used as tools for disease mechanism studies or drug screening. Svendsen and co-workers first generated iPSCs from a spinal muscular atrophy (SMA) patient and used them to derive motor neurons[191]. Amyotrophic lateral sclerosis (ALS) is another neurodegenerative disease involving motor neurons. Eggan *et al.* showed the possibility to produce motor neurons from iPSCs derived from patients suffering ALS mutations[192]. Similar achievements have also been made in other neurodegenerative diseases[193, 194].

5.2 Drug development with iPSCs

PD is the second most common neurodegenerative disease in man, yet there is still no effective therapeutic method for a cure. The most commonly used pharmaceutical therapy is L-DOPA[195], sometimes combined with dopamine receptor agonists. These methods are classical and effective in partially alleviating symptoms of patients suffering from PD. However, these drugs are not able to hold back disease progression. Gradually, patients become less sensitive to these pharmaceutical therapies and start to suffer from adverse effects, such as L-dopa induced dyskinesia[196].

For many years, the trial of drugs for PD therapy has relied on immortalized cell lines, animal models and then clinical trials. Fundamental research and preclinical research carried out in immortal cell lines and animal models are very crucial for effective drug discovery, but the interspecies differences make it hard to translate these readouts to the human disease. This ends up with an inefficient clinical trial when we try to test the drug on humans, because of the large difference that has to be overcome in this translational period. Thus, an efficient human species based model is highly sought after for the test of drugs for PD therapy.

IPSCs, with their self-renewability and potential to differentiate into any cell type of interest, shed positive light on cell replacement therapies for PD. Since pharmaceutical therapy cannot stop the process of dopaminergic neuronal death, iPSCs give hope to generating new dopaminergic neurons for autologous neural transplantation, as discussed in the section above. However, an alternative method of discovering new therapies is to explore new drugs that have better and longer lasting effects with fewer complications. Drug screening has been carried out mostly on cellular models such as modified immortal cell lines, primary neurons, and stem cells. Stem cells have proved useful tools for drug discovery. Unlike the other two types of cells mentioned previously, they can be maintained *in vitro* indefinitely, similar to immortalized cell lines, yet they do not involve any genetic modification. They can also be differentiated into neurons that are genetically and functionally

analogous to those of interest. Thus, compared to hESCs and neural stem cells, hiPSCs have clear advantages for drug screening.

Discovery of new therapy targets

Many drugs for treating neurodegenerative diseases work through certain signaling pathways. iPSCs provide a detailed view of disease mechanisms when used as a disease model and at the same time help researchers to reveal possible targets for chemicals or compounds to bind[197]. These kinds of targets can be protein or molecule binding, gene correcting or even direct biochemical treatments. iPSCs work as a window for us to see the treatment changes happening in the disease state. Researchers have been using zinc fingers as a genetic correction tool for treatment or study of familial PD, which is a genetic modulation method targeting gene mutation in PD[172]. There have been researches testing small compounds or growth factors in other neurodegenerative diseases such as ALS and AD. For example DHA has been tested in AD therapy, although this drug is not a newly found one, but the application of it on AD derived stem cells makes it possible for understanding the mechanism underlying the therapy[198]. In ALS, the chemical anacardic acid has been proven to revert pathological changes in ALS, such as shortened neuritis and reduced vulnerability to stress. Anacardic acid aims at mutant TDP43, which is a core pathological protein in ALS. Anacardic acid manages to clear the insoluble form of this protein[199]. In summary, using iPSCs as a tool to discover or test therapeutic targets is an efficient and also reliable approach for drug screening.

Personalized drug discovery

The variation of responses to drug therapy comes from differences in genetic background and also very diverse environmental influences. Epigenetic modifications and other genetic interruptions also contribute to varying drug reactions[200]. Thus, personalized drug discovery requires models that can reflect the variation of human individuals. The major advantage of PD patient derived iPSCs, compared to other cell types, is the accurate imitation of the individual patient, since iPSCs are generated from the individual patient's somatic cells with the same genetic background as those in

the patient's tissue. iPSCs may exhibit the same pathological changes as the patient's neurons, and the reaction to drugs may be similar too. One of the problems encountered when treating PD is the variation of therapeutic outcomes. Thus, personalized drug discovery appears to be very critical in this aspect.

The majority of PD cases are sporadic. Although studies have found several genes that indisputably contribute to PD onset, such as SNCA, LRRK2, and PINK1, PD is still a multifactoral disease. The etiology may come from several gene origins. No single animal model will be able to include all the genetic changes that may have happened to patients. The advantage of iPSCs here is that they comprehensively cover the genetic distribution in a patient or the diversity between different patients, which makes them perfect for personalized drug screening [201].

Potential for high-throughput drug screening.

PD is a multifactorial disease; the therapeutic targets are numerous. While some PD cases have defined genetic mutations or known environmental causes, in most cases, the cause remains unknown. Therefore, high-throughput drug screening is a useful approach to select drugs that are generally functional to most patients regardless of etiology. iPSCs provide the possibility to establish a library of disease specific iPSCs with or without gene mutations. The cells collected may come from an SNCA mutation patient, a PARK2 mutation patient or even a sporadic PD patient. This cell library may act as a group of sampled patients. It can represent the genetic and potentially environmental variation of a broad spectrum of the population. Using this library for drug screening gives a better prediction of how patients generally may react to a new drug and can also test the toxicity if necessary. At the same time, the screening offers insight into the underlying mechanisms as well when different iPSCs react differently to the treatment. The difference between a real patient group and an iPSCs library is that iPSCs exist in vast quantities and the number of new drugs that can be applied to this library is unlimited, while in a patient more than 3 or 4 drugs are almost beyond applicability[202, 203].

iPSCs as a tool to test drug toxicity

Side effects or toxicity have been a severe problem in PD therapy and most other diseases. Previously, toxicity tests were always carried out in animals or only discovered upon reaching last stage clinical trials, which brings up ethical problems and safety issues. Using iPSCs to test the toxicities of new drugs will decrease the number of animals being used and also, since they have a much larger potential for translation, their reaction is much closer to real patients. In a way, iPSCs provide a window for us to see into the mechanism of toxicity as well, which is essential for the improvement of drug discovery[204].

5.4 Establishment of biobank system for hiPSCs -- a direct comparison between autologous and allogeneic transplantation

Immune response plays a key role in the outcomes of transplantation. One of the major advances of hiPSCs is the syngeneic background with somatic cells. Autologous transplantation of iPSCs-derived cells compared to allogeneic transplantation results in a much lower immune response. Allo-recognition during transplantation will trigger the recipients' immune response thanks to the antigen or to the mismatch of major histocompatibility complexes (MHC), which decreases the survival of transplanted cells. And, at the same time, the recipient usually is under different combinations of immune suppression in order to maintain functionality of grafted cells[205]. Several studies have been looking into the immune response after autologous transplantation. Guha *et al* differentiate mouse iPSCs into three germ layers and upon transplantation into syngeneic rodents, they did not find any activation of the recipients' T cells or any antigen-triggered second immune response. They did not observe any rejection to syngeneic iPSCs from recipients[206]. Sundberg *et al* also differentiated human and non-human primate iPSCs into dopamine neurons. They improved the differentiation protocols by applying cell sorting based on neuronal markers. One year after autologous transplantation, one primate survived with iPSCs derived neural cells in the striatum with no trace of immune response[147]. Asuka *et al* did a direct comparison between autologous and allogeneic transplantation using primate

iPSCs; based on these studies, it appears evident that the autologous transplantation result in a much lower immune response compared to allogeneic transplants, which also leads to a higher ratio of dopaminergic cell survival after transplantation[205].

So, establishing a hiPSCs cell bank for the regenerative therapy of PD is very necessary. Unlike the transplantation of primate iPSCs, it is not realistic to establish a bank solely for autologous transplantations. Ideally, the cell bank should be able to provide at least HLA-matched iPSCs for patients with PD. Taylor *et al* reported the establishment of HLA-based iPSC cell bank matching 93% of the UK population by using hiPSCs generated from 150 volunteers[207]. Xue *et al have* developed a method for generating a large scale of hiPSCs from urine-derived cells in a feeder-free and virus-free method, which largely increase the possibility to establish this cell bank for clinical cell transplantation[208]. Similar achievements have been made by Beers *et al* by using xeno-free and feeder-free methods which allow to generate up to 200 lines per year[209]. All the above studies show us the necessity and also possibility of establishing a HLA based hiPSCs cell bank. This gives new hope for clinical cell transplantation therapy in PD.

5.5 Future challenges for hiPSC/hESC application

As discussed above, hiPSCs and hESCs have advanced as cell sources when applied in cell replacement therapy, disease modeling and regenerative medicine. In particular, hiPSCs, with their self-renewability and potential to differentiate into multiple somatic cell types, have been promising for the future study of PD. Recent improvements in neural differentiation and transplantation methods have made the application of hiPSCs more efficient and reliable.

However, problems still exist for further application of these stem cells. Firstly, using hiPSCs or hESCs for clinical therapy is still relatively unrealistic when it comes to both safety and ethical issues. The main ethical issue comes from hESCs sourced from human fetal tissue. In this aspect, hiPSCs solve the problem. However, hiPSCs bring up further safety issues. There exists evidence showing that hiPSCs might not be as similar to hESCs as previously thought when it comes to

differentiation potency and also the homogenization of differentiated cells. Neuronal differentiation of hiPSCs has significantly reduced efficiency and increased variability compared hESCs in the same condition in spite of the reprogramming method used[210]. This might be due to the instability of hiPSCs. We know that the reprogramming procedure is a site of introduction for exogenous genes. Although methods of eliminating transcriptional genes following the induction of iPSCs have been developed, without genomic monitoring, we still do not know how much influence the residual genes still have on the iPSCs. There are genomic analyses using high resolution SNP analysis that show differences in sub chromosomal copies between hESCs/hiPSCs and somatic cells. They also observe the duplication of oncogenes arising during the differentiation process, which is a further threat of tumor formation[211]. HiPSCs, further, have a different genetic profile to hESCs. Also, between passages, hiPSCs can be different from each other[212]. Somatic coding studies of iPSCs reveal the existence of several mutations after reprogramming, which are related to cancer formation as well[213]. All of these suggest that safety issues exist when it comes to the clinical use of both hiPSCs and hESCs. We need a completed system of gene monitoring before viably applying hiPSCs for cell therapy.

Besides the safety issue, there are other technical problems that must also be solved. As we discussed above, the low efficiency of dopaminergic neuron generation was one of the issues arising when it comes to cell transplantation. The recent improvement we described above partly solves the problem. However, a purer population of transplanted cells will still end up with better survival and less tumor formation. A method of purifying transplanted cells without the introduction of external marker genes, such as fluorescent proteins, is in high demand. As for now, most of the purification before transplantation relies on FACS sorting, which inevitably involves genes such as GFP[214].

6 Perspective

Here, we have summarized recent developments regarding the application of iPSCs and ESCs in PD research, following the line of: hiPSCs/hESCs → neural progenitors → neural progenitor with dopaminergic identity → transplantation. Many remarkable achievements have been made. We are currently at a stage of generating dopaminergic neurons from PD derived-iPSCs using relatively rapid and feeder cell-free, and most importantly, more highly efficient protocols, which are based on a better understanding of the molecular mechanisms. The grafting of dopaminergic neurons or neural progenitors can achieve an outcome of tumor-free transplants with high survival and integration rates. This will shed light on future clinical applications of iPSCs.

However, we are still facing major problems. In the future, iPSCs may be mainly used in two different ways related to PD, for cell replacement therapy in curing PD and as a model for drug screening and disease mechanism studies.

For cell replacement therapy, a ¨clean¨ genetic background and high purity level will be necessary for transplantable cells. Although a protein based cell-reprogramming method has been reported, its low efficiency in generating iPSCs hinders its usefulness for large-scale applications. Most of the iPSCs in use are still generated using traditional methods such as virus transduction or cDNA transfection. To generate iPSCs with a completely clean genetic background to be used in cell therapy, we must increase the efficiency for reprogramming using a protein-based method.

Although the efficiency has been dramatically increased, the challenge on purity of the cells for transplantation remains. Cells used for transplantation are expected to have a homogenous population, which will give a consistent outcome and no risk of tumor formation in the long run. The same expectations also apply for iPSCs-derived neurons that are used for disease mechanism studies and drug discovery.

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