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Dopaminergic Differentiation Using Pluripotent Stem Cells

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ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disorder. The motor symptoms of PD are caused by the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta of mesencephalon. The causes for death of DA neurons are not well understood, but the strongest risk factor is increasing age. There is no cure currently available for PD, and treatment is limited to management of PD symptoms in patients. Primary DA neurons are virtually unobtainable from living patients and animal studies have proven inadequate for studying the mechanism of PD development. Pluripotent stem cells (PSC) are primary self-renewing cells capable of differentiating into all cell types of an organism, including DA neurons. PSCs represent an abundant source of cells that can be genetically modified or isolated from patients with complex diseases, enabling the production of large quantities of DA neurons for disease modeling, drug screening, and gene function studies. Furthermore, since PD arises as a result of deterioration of DA neurons in a specific brain region, it has been suggested that a relatively small number of cells could restore normal function. PSCs could provide a source of DA neurons for cell replacement therapy. In this Prospects article, we focus on the development and in vitro derivation of DA neurons from PSCs, as well as current applications of the technological advances, with the emphasis on future directions and efforts in the field. J. Cell. Biochem. 113: 3610–3619, 2012.

KEY WORDS: PARKINSON'S DISEASE; DOPAMINERGIC NEURONS; DIFFERENTIATION; PLURIPOTENT STEM CELLS; DISEASE MODELING; CELL THERAPY

Within the last century, advances in medicine and technology have led to longer life expectancy. Along with increased longevity, there has been an increase in the incidence of age-related neurodegenerative diseases. Parkinson's disease (PD) is one such neurodegenerative disease in which the dopaminergic (DA) neurons localized in the pars compacta substantia nigra progressively deteriorate. PD is the second most prevalent neurodegenerative disease, next to Alzheimer's disease, affecting approximately 0.5% of adults 65–69 years old [Tanner and Goldman, 1996].

The affected DA neurons are responsible for movement control. Hence, the disruption of the transmission of signaling caused by DA neurons' deterioration results in an array of defects in motor control. PD patients often suffer from symptoms such as a fixed inexpressive face, an unsteady gait, postural instability, a resting tremor, muscle weakness, and slow rigid muscle movements. These motor dysfunctions strongly affect the quality of life of afflicted individuals, as these patients undergo massive loss of strength, balance, manual dexterity, and the ability to easily initiate movements.

Although there are medications used to alleviate symptoms of PD, such as levadopamine, the numerous side effects, dosage consistency, and limited delivery effectiveness leave patients in a great deal of discomfort and unease. Levadopa is a commonly administered drug-therapy of PD. It is a precursor of dopamine that, unlike dopamine, can cross the blood brain barrier and is enzymatically converted to dopamine in neurons. Levadopa is an imperfect drug with severe side effects, including involuntary muscle movements, such as undulating movements, grinding of the teeth, protrusion of the tongue, twisting, twitching, and akinesia, or the sudden inability to move. In some cases, levadopa has gastrointestinal and cardiovascular side effects, such as ulcers, hemorrhaging, nausea, vomiting, and arrhythmias. Other side effects include changes in mental health states, mood cycling, psychosis, hallucinations, anxiety, and insomnia, possibly due to the desensitization of dopamine receptors [Pearce, 1984]. Thus, two

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stages of the disease are usually distinguished—an initial stage when patients develop symptoms that require pharmacological intervention, and the second stage in which additional complications develop due to treatment with medication and disease progression. Surgery, such as, deep brain stimulation may be used as well—either in combination with levodopa when it is not sufficient, or alone when levodopa side effects overcome its benefit. Pharmacological and surgical approaches aim to control the symptoms of PD, but do not eliminate the root cause of the disease, that is the loss of DA neurons. The alternative approach to treatment of PD is substitution of lost DA neurons. In first attempts of cell transplantation, fetal tissue was used as a source of dopamine producing cells. Some patients showed remarkable response, but others developed graft-induced dyskinesia, as well as other side effects. Additional obstacles associated with the use of human fetal tissue for cell derivation include scarcity, genetic diversity, inconsistent gestation age, as well as ethical concerns. Together these problems challenge the feasibility of cell therapies based on transplantation of dopamine producing cells derived from fetal tissue; hence other sources of dopamine neurons are needed.

The answer may lay in pluripotent stem cells (PSCs), which are self-renewing cells with the propensity to give rise to all cell types within an organism. There are different sources of PSCs, such as embryonic stem cells (ESCs), embryonic germ cells (EGCs), embryonic carcinoma cells (ECCs), epiblast stem cells (EpiSCs), and induced pluripotent stem cells (iPSCs). ESCs are isolated from the inner cell mass of the blastocyst stage embryo and can be grown in vitro for prolonged periods of time. In 2006, Shinya Yamanaka discovered a novel way to derive PSCs [Takahashi and Yamanaka, 2006]. By introducing a combination of genes, somatic cells could be reprogrammed into a pluripotent state [Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007]. These cells share numerous characteristics of ESCs, including morphology, expression of pluripotency markers, and ability to differentiate into three germ layers, and are referred to as iPSCs. Subsequently, it was shown that iPSCs can be generated from a variety of tissues and species by driving the ectopic expression of a few defined transcription factors. The Yamanaka cocktail defines *POU5F1 (OCT4)*, *SOX2*, and *KLF4* (OSK) as essential. Other combinations of transcription factors are also capable of inducing pluripotency. *LIN28*, *NANOG*, *OCT4*, and *SOX2* were successfully used by the Thomson group to derive human iPSCs [Yu et al., 2007]. *OCT4*, *SOX2*, and *NANOG* are core transcriptional regulators that are essential for the maintenance of pluripotency. *KLF4* is involved in cell proliferation, differentiation, survival, as well as transcriptional activation and repression of specific domains. *LIN28* is involved in the inhibition of differentiation-inducing microRNAs such as LET7 family. Originally, Yamanaka's cocktail included *cMYC*, a known oncogene that plays a role in regulation of the cell cycle and cell proliferation. Inclusion of *cMYC* significantly improved the efficiency of reprogramming, but increased tumorigenic potential of iPSCs. Introduction of reprogramming factors into somatic cells can be achieved with a variety of vectors. The original method relied on the use of integrating vectors, such as lenti or retro viruses. However, this approach raised the risk of transgene reactivation and caused insertional mutagenesis in iPSCs. Alternatively, reprogramming factors may be delivered by non-integrating plasmids, or as recombinant proteins, but this results in lower efficiency of reprogramming. Depending upon the ultimate fate and usage of the iPSCs, an appropriate method of derivation and source of cells must be considered.

Remarkably, researchers are able to grow PSCs in defined conditions that optimize the differentiation of these cells to any desired cell within the human body. The ability to utilize stem cells as a tool to better understand and treat degenerative diseases is vastly improving due to the innovation and dedication of scientists around the world. Early research has shown the ability to derive DA neurons from PSCs which may be used in drug screening and cell replacement therapy in PD models. iPSCs have an advantage over ESCs, as they may be used to derive patient-specific neurons, including neurons with genetic mutations incorporated into the genome. iPSCs present the opportunity to study the disease and perform drug screening more accurately, as well as implement autologous cell replacement therapy more efficiently.

In this Perspective article, we will discuss the earliest stages of development of the nervous system, that is, the processes of specification of neural epithelium and its subsequent diversification into region-specific neurons and glia, followed by a summary of midbrain development and the emergence of DA neurons. In the last section, we will focus on new technologies for isolation, genetic modification, and differentiation of PSCs and their applications. In general, the description of in vivo observations will be followed by the summary of recent in vitro findings in PSCs.

EARLY NEURAL DEVELOPMENT

NEURAL INDUCTION

During embryogenesis, the first step in the development of the nervous system is neural induction, the specification of dorsal ectoderm to become neural epithelium. In the early 1920s, Spemann and Mangold discovered an "organizer" in Xenopus embryos capable of inducing neural fate in dissected ectoderm. Subsequently, similar organizers were discovered in fish (embryonic shield), birds (Hensen's node), and mammals (gastrula organizer/the node in mice), suggesting that neural induction is, at least in part, conserved among vertebrates. More recent studies began unveiling the molecular pathways through which the organizers instruct dorsal ectoderm to adopt neural fate. Studies in Xenopus and zebrafish embryo development demonstrated that the absence of repressive influences of WNT and of TGFβ superfamily members, BMP and NODAL, are sufficient for specification of neural epithelium (Fig. 1) [Levine and Brivanlou, 2007]. These experiments led to the proposal of the so-called default pathway for neural induction—neural fate is a default state in the ectoderm, and TGFβ/BMP signaling is necessary to prevent neural induction in non-neural regions of the ectoderm [Levine and Brivanlou, 2007]. Thus, the role of an organizer is to express antagonists of BMP, NODAL, and WNT [Gaulden and Reiter, 2008].

As predicted by the default pathway of neural induction, one of the most readily derived lineages from ESCs was neural. Culturing of mouse ESCs at low density, thereby averting cell to cell communication, and in the absence of any extrinsic signals resulted

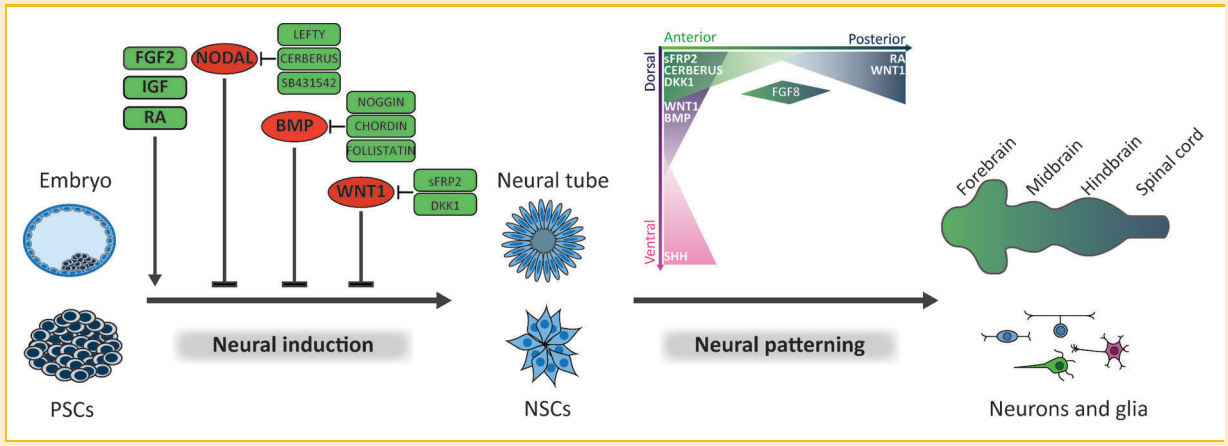

Fig. 1. Neural induction and patterning during in vitro PSC differentiation reflect in vivo neural development. Neural induction is initiated by repression of BMP, NODAL, and WNT signaling in dorsal ectoderm. Recent evidence suggests that FGF2 and IGF exert a positive effect. RA does not have a known function in in vivo neural induction, but promotes neural induction in mouse ESCs. In human ESCs, RA has a posteriorizing effect. Anterior–posterior and dorsal–ventral axes are established in the orthogonal gradients of signaling factors, resulting in the partitioning of neural tube in discrete regions.

in the expression of neural markers within several hours [Smukler et al., 2006]. Furthermore, inhibition of BMP/TGFβ and WNT signaling potentiated mouse and human ESC differentiation into neural lineages, providing additional support for the default pathway as a model of neural induction [Kawasaki et al., 2000; Ying et al., 2003; Chambers et al., 2009]. In addition to inhibition of repressive influences of BMP and WNT, mounting evidence suggests the existence of positive regulation of neural induction by FGF signaling in vivo, at least in amphibians and birds [Gaulden and Reiter, 2008]. FGF and IGF signaling appears to be a potent neural inducer in mouse and human ESCs [Zhang et al., 2001; Smukler et al., 2006]. The exact role of FGF signaling is unknown, but it has been proposed to act as a "competence factor" for neural induction in pre-gastrulating mouse embryo and as a "maintenance factor" in neural tissue later on [Levine and Brivanlou, 2007]. FGF and IGF could act to inhibit BMP/NODAL signaling at the intracellular level. At the molecular level, the convergent point of FGF/IGF and BMP/NODAL signaling is SMAD1 [Gaulden and Reiter, 2008]. BMP and NODAL signaling via BMP receptor serine/threonine kinase promote phosphorylation of the carboxyl terminus and nuclear localization of SMAD1. FGF and IGF signaling through a receptor tyrosine kinase and ERK kinase result in the phosphorylation of the linker region and inhibition of SMAD1 transcriptional activity. Thus, BMP antagonists prevent activating phosphorylation of SMAD1, whereas FGF and IGF induce inhibitory phosphorylation of SMAD1 and enhance intracellular inhibition of BMP signaling.

NEURAL PATTERNING

Following neural induction, neural tissue undergoes a series of differentiation and morphogenetic processes to form distinct regions of the central nervous system (CNS); this process is termed neural patterning. In the 1950s, Nieuwkoops proposed "activation and transformation," a two-stage model for neural development. According to this model, nervous tissue is induced (activated) in the dorsal ectoderm and is of anterior identity early on; anterior neural tissue then undergoes "posteriorization" to form midbrain, hindbrain, and spinal cord (transformation).

How these processes occur in vertebrates is a complex question that has captivated scientist for decades after early discoveries made by Spemann and Mangold. The function of an organizer in induction and organization of the neural tube in lower vertebrates is conferred upon three distinct derivatives of a gastrula organizer (GO) and an extraembryonic tissue in mice. The GO expresses multiple BMP inhibitors and is necessary for induction of the first neural tissue with anterior character during early to mid-streak stages (E6.5–E7 in mouse embryonic development). The importance of the GO is underscored by the observation that the only mouse mutants that completely fail to induce neural tissue are those that lack the GO. Newly formed anterior neural tissue moves away from the GO to become juxtaposed with anterior visceral endoderm (AVE) that protects its anterior identity. AVE cannot induce neural tissue in explants, but secretes CEREBRUS and LEFTY that inhibit NODAL signaling and suppress the expression of markers of posterior tissues. The GO subsequently forms morphologically distinct structure termed the node, which is required for the induction of caudal neural tissue. The node retains neural inducing activity of its predecessor, but is localized in the vicinity of posteriorizing factors. The final GO derivative, the anterior mesendoderm (AME) at the late streak stage (E7.5–E8.0) maintains anterior neural tissue through the BMP inhibitory mechanism and is required for forebrain development [Levine and Brivanlou, 2007].

The neural tube is partitioned into regions of cells with discrete fates along dorso-ventral and anterior–posterior axes based on cellular position in the gradient of signaling molecules (Fig. 1). The dorso-ventral axis is established by the gradient of Sonic hedgehog (SHH), WNT, and BMP molecules. SHH is a specifying factor of the ventral neurons and is expressed on the ventral side along the entire length of the neural tube. Its strong ventralizing activity is repressed by BMP and WNT signaling on the dorsal side of the neural tube. As already described, the early neural tube is of anterior identity, and

posteriorization occurs subsequently in the presence of caudalizing factors such as retinoic acid (RA), FGF, and WNT. Diversification of early CNS progenitors by signaling molecules is not only concentration dependent, but also temporally controlled; in general, neurogenesis precedes gliogenesis during the development. This was recapitulated in vitro using ESC-derived neural stem cells (NSCs). The ESC-based assays enabled identification of key transcriptional factors responsible for the gliogenic switch and temporal specification of NSCs. Chicken ovalbumin upstream promoter transcription factor I and II (COUPTFI and II, also known as NR2F1 and 2) act primarily by regulating epigenetic silencing of gliogenic genes. Also, committed neuronal precursors express NOTCH ligands that activate NOTCH signaling on neighboring NSCs resulting in the expression of nuclear factor I transcription factor and expression of astrocyte specific genes [Gaspard and Vanderhaeghen, 2010]. Thus, it appears that committed neuronal precursors and young neurons promote differentiation of the remaining uncommitted progenitors into glial cells.

The differentiation of ESCs in vitro follows the same developmental principle: the ESC-derived NSCs exhibit anterior identity early on, and can be directed to posterior fates by the addition of signaling molecules. For example, neural tube-like rosettes arise from ESCs and express forebrain markers OTX1, PAX6, EMX2, GSH2, and DLX2 [Bouhon et al., 2006]. Following addition of FGF2 and RA, these cells acquired first midbrain (EN2), and then hindbrain (EGR2) characteristics [Bouhon et al., 2006], whereas RA and SHH promoted differentiation into motoneurons [Bouhon et al., 2006]. Similar to the findings in neural plate explants [Ye et al., 1998], SHH and FGF8 specified midbrain DA differentiation of ESCs in vitro [Lee et al., 2000]. However, neural progenitors demonstrate increased gliogenic bias, decreased neurogenic potential, and are refractory to extracellular signals after prolonged culture. This observation suggests that the regionalization of ESC-derived NSCs may be temporally restricted depending on the culture conditions. Taken together, these observations indicate that neural induction and regional neural specification in ESCs recapitulate the in vivo development of the neural tube. Next, we will focus on midbrain DA neurons that are the affected cell type in PD patients.

DOPAMINERGIC NEURONAL DEVELOPMENT

Dopaminergic (DA) neurons are present in several brain regions, including the olfactory bulbs, the hypothalamus, and the retina, but the most prominent groups of DA neurons reside in the mesencephalon (midbrain). Two distinct clusters of DA neurons can be distinguished in the midbrain: A9 neurons in the substantia nigra pars compacta and A10 neurons in the ventral tegmental area. The A10 cluster projects into the ventral striatum and limbic structures and is involved in novelty and reward systems. The A9 neurons are located relatively lateral to the midline, project into the striatum, and regulate the extrapyramidal motor system that controls postural reflexes and initiation of the movement. The underlying pathophysiology of PD is the loss of A9 DA neurons in the midbrain.

MIDBRAIN DEVELOPMENT

Cell mapping studies suggest that multipotent mesencephalic progenitors develop from the floor plate, a region of mesencephalon at the ventral midline near the midbrain–hindbrain boundary (MHB; also known as isthmic organizer). Mesencephalic progenitors go through four stages en route to mature DA neurons: (1) adoption of committed DA neuronal precursor fate; (2) exit from the cell cycle; (3) expression of early DA markers; and (4) maturation and establishment of connections with other neurons. This process is directed by an intrinsic transcriptional network and instructive cues from two signaling centers—the midline floor plate and the isthmic organizer. The midline floor plate and the isthmic organizer secrete diffusible factors SHH and FGF8, respectively, which form orthogonal concentration gradients [Ye et al., 1998]. Intersection of the physical location of progenitors in the SHH and FGF8 gradients with the intracellular transcriptional network determines fate choices and acquisition of DA neuronal identity. Studies using naïve neuroepithelial explants and in vitro derived neuroepithelial progenitors demonstrated that SHH and FGF8 are sufficient for induction of DA fate in these cells [Ye et al., 1998]. Other secreted signaling molecules play a role in DA neuronal development, too. Loss of WNT1 at early stages of mouse midbrain development (E9.5–E10.5) results in the absence of midbrain DA neurons and the ectopic generation of rostral hindbrain serotoninergic neurons [Prakash and Wurst, 2006]. WNT1 expression is also required at the time when post-mitotic DA precursors begin to differentiate (E11.5–E12.5) [Prakash et al., 2006].

TRANSCRIPTIONAL REGULATION OF DOPAMINERGIC NEURON DEVELOPMENT

As mentioned above, midbrain DA progenitors go through multiple steps during differentiation into mature neurons. Each step is regulated by a set of transcriptional regulators which are activated by extrinsic signaling factors (Fig. 2). In dividing mesencephalic progenitors (E9.5–E10.5), SHH signaling via its receptor PATCHED induces GLI2a and 1 transcriptional activators and suppresses GLI3 transcriptional repressor. GLI1 activates expression of the floor plate marker *FOXA2*, while suppression of GLI3 relieves *FGF8* repression. FOXA2 induces expression of pro-neural transcription factor neurogenin 2 (*NGN2*) and of *SHH*, thereby creating a positive feedback loop. Both SHH and WNT1 induce expression of LIM homeobox transcription factor alpha (*LMX1A*) in mouse floor plate cells. LMX1A activates the expression of *NGN2* and muscle segment homeobox 1 (*MSX1*) repressor that inhibits negative regulators of neurogenesis such as *NKX6.1*. LMX1A also induces *WNT1* expression, closing the positive feedback loop. Orthodenticle homeobox 2 (OTX2) transcription factor is also involved in DA neuron development as evidenced by severe reduction in mesencephalic DA neurons caused by *OTX2* conditional deletion in mice. OTX2 represses a negative regulator of DA development *NKX2.2*, and promotes activation of pro-neural genes *MASH1* and *NGN2*. Overexpression of *LMX1A*, *OTX2*, and *FOXA2* strongly promotes DA differentiation, confirming that the synergistic activity of these transcription factors plays a central role in DA development [Prakash and Wurst, 2006; Abeliovich and Hammond, 2007].

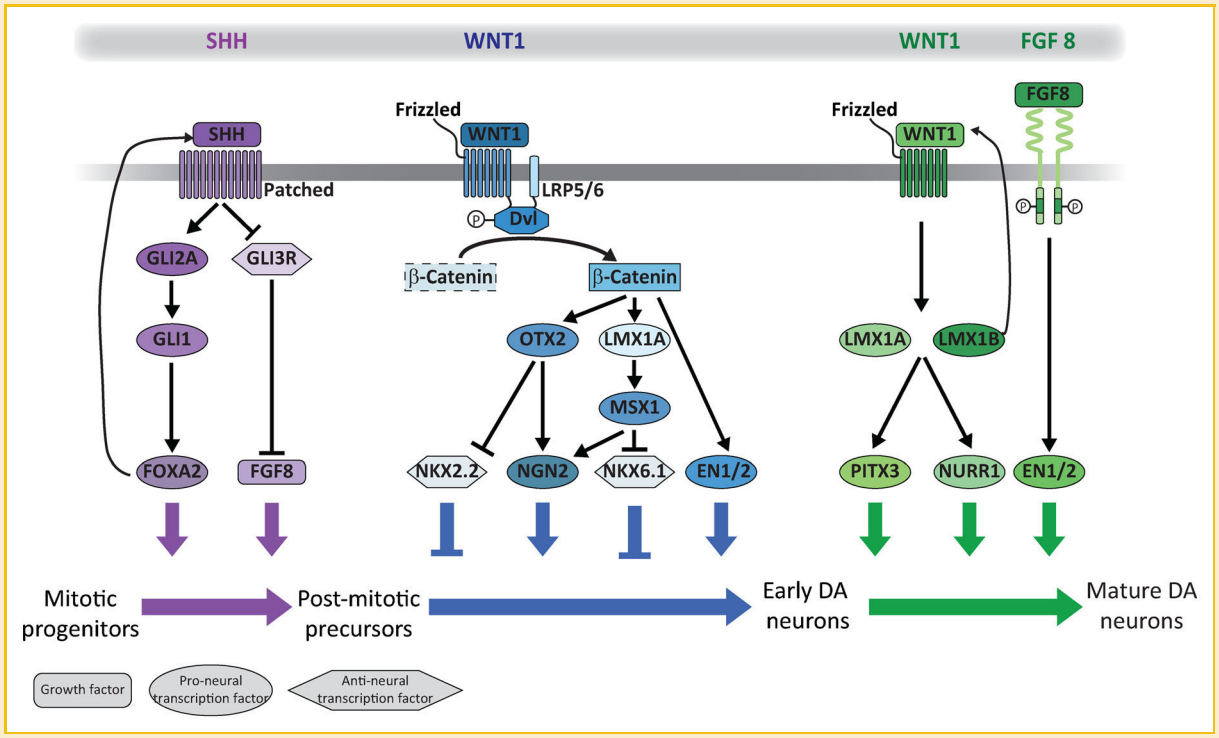

Fig. 2. Transcriptional regulation of midbrain dopaminergic differentiation. Midbrain DA precursors arise from the floor plate in the presence of SHH, FGF8, and WNT1. These growth factors activate a cascade of transcriptional regulators that govern differentiation of midbrain DA precursors into mature DA neurons. The time scale is based on mouse embryonic development.

Pro-neural gene *NGN2* expression is maintained in post-mitotic DA precursors, suggesting its role in neuron maturation in addition to its role in dividing precursors. As DA precursors exit the cell cycle, they migrate from the ventricular surface and begin maturation. At this time (E10.5–11.5), the expression of enzymes involved in dopamine synthesis, such as tyrosine hydroxylase (*TH*), can be detected. Dopamine transporter (*DAT*) expression occurs at E12–15 and is more specific than TH to mesencephalic DA neurons. Transcriptional network composed of *NURR1*, *LMX1B*, *PITX3*, and *EN1/2* is involved in the determination of the DA phenotype in post-mitotic precursors. *LMX1B* mutant mice express *NURR1* and *TH* early on, but fail to express *PITX3*, placing LMX1B upstream of PITX3. Furthermore, LMX1A and LMX1B exhibit significant functional overlap. Both proteins can bind to *WNT1*, *NURR1*, *PITX3*, and *MSX1* promoters, as well as to each other's promoters, indicating cross-regulation between LMX1A and LMX1B. Engrailed 1 and 2 (*EN1/2*) are required for generation of isthmus and are initially expressed in this region. Subsequently, *EN1* and *EN2* are expressed specifically in post-mitotic midbrain DA neurons and are necessary for their late maturation and survival [Prakash and Wurst, 2006; Abeliovich and Hammond, 2007].

IN VITRO DERIVATION OF DOPAMINERGIC NEURONS FROM PSCS

Numerous protocols with varying degrees of efficiency for derivation of DA neurons from PSCs are in use. These protocols can be grouped in three major categories: (1) default pathway methods that rely on an intrinsic program and spontaneous differentiation of PSCs followed by subsequent selection of desired cell type; (2) co-culture with PA6 or MS5 mouse stromal cells that promote DA differentiation; and (3) differentiation in chemically defined medium. Neurons can also be derived by direct lineage conversion of terminally differentiated cell types. Different approaches for the derivation of DA neurons are summarized in Table I. Default pathway methods are non-specific and require multiple steps for the isolation and enrichment of a desired cell type. Co-culture methods involve the use of mouse stromal cells, introducing the risk of xeno-contamination of DA neurons. Neither method allows for the study of the role of various signaling molecules in differentiation of PSCs without confounding effects of spontaneous differentiation or signaling factors produced by stromal cells. Thus, there is a great need for a protocol that allows the derivation of neural progenitors/stem cells in defined conditions. Recently, several studies reported methods that promote neural differentiation of human ESCs in defined conditions. Human PSCs are induced to form neuroepithelial cells by inhibition of BMP and TGFβ signaling pathways and are expanded in the presence of FGF2, whereas midbrain patterning is subsequently achieved with FGF8 and SHH.

The elucidation of the signaling pathways that direct the differentiation of midbrain DA neurons has been employed in improving the efficiency of protocols for derivation of DA neurons from PSCs. For example, two groups demonstrated that *Nurr1*-expressing mouse ESCs generate TH^+ cells with molecular,

TABLE I. Methods for Derivation of DA Neurons

Key features	Efficiency	Characterization	Reference
Co-culture methods			
Neural induction by co-culture of mouse ESCs with stromal feeders	52% neurons, 30% of which are TH^+ cells	Expression of DA markers, transplantation into mouse brain	Kawasaki et al. [2000]
Neural induction by co-culture of primate and human ESCs with stromal feeders	80% TH^+ colonies	Expression of DA markers, transplantation into PD rats	Kawasaki et al. [2002] and Zeng et al. [2004]
Neural induction by co-culture with stromal feeders followed by the application of defined growth factors for neural patterning and DA selection	30–50% TUJ^+ neurons, of which 64–79% are TH^+ cells	Expression of DA markers, electrophysiology	Perrier et al. [2004]
EB-based methods			
Five step protocol from mouse ESCs	34% of neurons are TH^+ (22% of all cells)	Expression of DA markers, electrophysiology	Lee et al. [2000]
From human ESCs in the presence of FGF2	96% $Nestin^+$ cells, few TH^+ cells	Expression of neural markers, transplantation into neonatal mouse brain	Zhang et al. [2001]
Five step protocol from mouse ESCs overexpressing pro-DA neuron transcription factor with or without growth factors	50% TH^+ without SHH/FGF8, 78% TH^+ with SHH/FGF8	Expression of DA markers, electrophysiology, transplantation into PD rats	Kim et al. [2002]
Neural induction in EBs followed by the application of defined growth factors for neural patterning and DA selection	50–60% of TUJ^+ cells are TH^+	Expression of DA markers, transplantation into PD rats, electrophysiology	Swistowski et al. [2010] and Yan et al. [2005]
Default pathway			
Serum-free, EB-free, extracellular signaling-free differentiation along neural lineages	>90% $Nestin^+$, $SOX1^+$ cells	Expression of neural markers	Smukler et al. [2006]
In the presence of FGF2, EGF, N2, and/or B27 to promote neural induction and expansion of early neural progenitors	99% $NCAM^+$, 97% $Nestin^+$, <1% TH^+	Expression of neural markers, transplantation into neonatal mouse	Reubinoff et al. [2001] and Ying et al. [2003]
In the presence of inhibitors of TGFβ superfamily member signaling and/or SMAD inhibitors to prevent non-neural ectoderm fates, followed by neural patterning with SHH and FGF8 to induce DA neurons	>80% $PAX6^+$	Expression of neural and DA markers	Chambers et al. [2009] and Zhou et al. [2010]
Direct conversion			
Direct conversion of non-neural cell types to induced neurons by the forced expression of key transcription factors	Up to 60% TUJ^+, no TH^+, no $EN1^+$ cells	Expression of neuron markers, electrophysiology	Pang et al., [2011] and Vierbuchen et al. [2010]

morphological, and physiological characteristics of authentic midbrain DA neurons more efficiently than wild type mouse ESCs. *Nurr1* expression not only enhanced the yield of midbrain DA neurons, but apparently their in vivo function as well. Overexpression of *Nurr1* resulted in the upregulation of all DA midbrain markers (A9 and A10), and the increase in total number of DA neurons. However, *Pitx3* overexpression in mouse ESCs increased the yield of A9 neurons, while the total number of DA neurons did not change. Therefore it appears that *Nurr1* affects overall midbrain DA neuron differentiation, whereas *Pitx3* has a role in specification and maintenance of A9 DA neurons. Similarly, another study showed that *Lmx1a* overexpression in mouse ESCs resulted in robust generation of midbrain DA neurons. In human ESCs, overexpression of transcription factors previously identified to play a role in the development of midbrain DA neurons revealed that these factors cannot induce mature DA neurons independently. However, another group demonstrated that extrinsically expressed *NURR1* and *PITX3* can cooperatively promote differentiation of human and mouse ESCs into mature DA neurons [Abeliovich and Hammond, 2007; Gaspard and Vanderhaeghen, 2010; Gaulden and Reiter, 2008].

The ability to alter the fate of a terminally differentiated cell raised another important question: would it be possible to directly convert one cell type to another, and could we produce neurons from other cell types directly and bypass the iPSC stage? The answer is yes. Functional neuronal cells (induced neurons, iNs) were obtained by ectopic expression of *ASCL1*, *BRN2*, and *MYT1L* in mouse [Vierbuchen et al., 2010], and human [Pang et al., 2011] fibroblasts. Expression of two transcription factors (*MYT1L* and *BRN2*) and microRNA (miR-124) directly reprogrammed human fibroblasts into iNs. In all cases, iNs exhibited neuronal morphology, gene expression profile, fired action potential, and formed functional synapses. However, none of these studies reported generation of TH-positive, midbrain DA neurons. Although these results demonstrate that the direct reprogramming of adult terminally differentiated cells into functional neurons is feasible in defined conditions, they do not alleviate the need to reprogram adult cells into iPSCs. Direct lineage conversion is extremely inefficient, and, unlike protocols with iPSCs, does not enable production of large quantities of a desired cell type. Following reprogramming of the adult cells, the iPSCs can be expanded and stored long term, genetically modified, or differentiated into any cell type. Direct conversion of cellular fate can be a faster process because the intermediate iPSC step is omitted, but results in a lower yield of the desired cell type and iNs lack the plasticity of iPSCs.

iPSC-DERIVED DOPAMINERGIC NEURONS

Recent advancements in induced pluripotency technology led to proliferation of research aiming to adopt ESC growth and differentiation protocols to iPSCs. Induced PSCs are free of ethical concerns that limit ESC research, yet possess ESC's self-renewal and differentiation capacities. Since iPSCs can be derived from affected and healthy individuals, and can adopt any specialized cellular fate, they provide ways for studying human development and understanding of disease processes in vitro that have not been available

before. Importantly, PSCs play a central role in conception of cellular therapies and personalized medicine.

iPSC-derived DA neurons as research tools. The prospects of cell replacement therapy have mostly captivated the public and aided in acquiring support for PSC research, but cellular therapies require much work before fruition. In the realm of current and future application of iPSC technologies, less publicized, but equally valuable is the use of iPSCs as research tools.

Disease modeling. PD symptoms manifest only after the death of the majority of A9 DA neurons, suggesting that the underlying pathophysiology is well under way for years, and likely decades, prior to the onset of symptoms. It is reasonable to propose, therefore, that the most effective therapeutic strategy would be the prevention, or slowing down, of the loss of A9 DA neurons in patients before any symptoms develop. However, we are only beginning to understand the causes of the death of DA neurons in the midbrain of PD patients, and without knowledge of the cellular events that lead to death of DA neurons, it will be impossible to devise therapies to block those processes. Thus far, very little is known about PD mechanisms at the cellular level, besides the fact that DA neurons are dying. This is an area of research where iPSCs may prove invaluable, as iPSC-derived neurons can be used as disease models to test new hypotheses regarding the mechanisms and sources of toxicity in DA neurons.

Genetic studies have aided in the identification of a growing list of genes involved in the etiology of PD, and are the subject of multiple reviews [Obeso et al., 2010; Wider et al., 2010]. Leucine rich repeat kinase 2 (*LRRK2*) is likely the most documented gene associated with PD. The most common mutation in *LRRK2*, *G2019S*, is found in about 10% of familial and 3% of sporadic cases. Other examples include alpha-synuclein (*SNCA*), which is mutated in about 2% of familial cases, parkin (*PARK2*), *PINK1* (*PARK6*), and *DJ1* (*PARK7*). The common denominator for these proteins is that they are involved in mitochondrial, endoplasmatic reticulum, and/or Glogi apparatus function, suggesting that oxidative damage and aberrant protein metabolism (protein folding, localization, and proteosomal degradation) are the underlying causes of toxicity in the cells carrying these mutations. Unavailability of the primary neurons from PD patients prevented studies on the exact mechanisms of toxicity, and the majority of gene function studies were done on fibroblast cell lines transfected with mutated genes. Despite efforts in discovering genetic causes of PD, the majority of cases are idiopathic, making it even more difficult to understand the cause(s) of cytotoxicity. Finally, there is growing evidence that PD is a group of diseases with similar symptoms caused by overlapping, yet distinct mechanisms.

Reprogramming of cellular fate enables scientists to generate patient-specific iPSCs and differentiate them into authentic A9 DA neurons for modeling PD at the cellular level. For example, iPSC lines have been derived from patients harboring *LRRK2 G2019S* mutation [Nguyen et al., 2011], alpha-synuclein triplication [Byers et al., 2011; Devine et al., 2011], *PARK2* mutations [Jiang et al., 2012], as well as from patients with sporadic PD [Soldner et al., 2009]. One of the hallmarks of PD at the cellular level is the formation of alpha-synuclein and ubiquitin protein aggregates called Lewy bodies, which was recapitulated to a certain degree in DA neurons generated from patient-specific iPSCs carrying *SNCA* triplication. Similarly, *LRRK2* mutant iPSC-derived DA neurons exhibit an increased susceptibility to oxidative stress, and an increased expression of oxidative stress response proteins and of *SNCA*. Thus, neurons obtained from patient-specific iPSCs carrying different PD-associated mutations may provide a missing link between current models of disease mechanism and actual PD pathophysiology, as well as help identify new measurable phenotypes as therapeutic targets.

PD is a multifactorial disease that results from a complex interaction between genotype and environment. Environmental marks are translated to the particular epigenetic modifications of the genome, which are highly heritable. Thus, genotype, environment, and epigenetic landscape all contribute to disease pathology. During the reprogramming, epigenetic marks acquired during ontogeny are stripped as cells are reverted to the pluripotent state, rendering it difficult to accurately reproduce disease phenotype in iPSC models of complex diseases, unless epigenetic changes are well known and can be recreated in iPSCs [Cherry and Daley, 2012]. Nevertheless, generation of iPSCs from patients with idiopathic PD may be helpful because the genetic profile that predisposes an individual to PD directly, or to acquiring epigenetic variability and the disease indirectly, is faithfully transmitted to the iPSCs.

Drug screening. Unveiling the disease mechanisms and relevant therapeutic targets can also improve designing new therapies, particularly in light of recent findings that document genotype-specific disease phenotype in some disorders. For example, researchers discovered that long QT syndrome can be caused by mutations in two different genes, for both of which the cellular models now exist owing to the iPSC technology. This enables scientist to directly compare cellular phenotypes between two different genotypes of the same disease and develop better therapies to target the long QT syndrome in patients with two different mutations [Cherry and Daley, 2012]. Similarly, it is possible to envision that PD patients with Lewy bodies and those without will respond differently to therapeutic agents, and each patient might require different treatment. Therefore, generating a pool of iPSCs carrying different disease-associated mutations would create a powerful platform for drug screening. As mentioned above, not only that many genes are identified in familial cases of PD, but also multiple variants in the same gene are associated with the disease. The ability to generate iPSCs and DA neurons from patients carrying different mutations in *SNCA*, *LRRK2*, and *PARK2*, to mention just three most studies PD-associated genes, would allow for screening of thousands of chemical compounds in order to identify the ones with the greatest effect for each individual variant. Indeed, there is a worldwide effort, including our laboratory, to generate iPSC lines from PD patients carrying mutations in *SNCA*, *LRRK2*, *PARK2*, and other PD-associated genes, as well as from sporadic cases. We believe that iPSCs and DA neurons will be invaluable for toxicity and neuroprotective drug screening.

Genetic modification of iPSCs. Ability to genetically manipulate iPSCs in vitro additionally diversifies iPSC utilization. For example, introduction of reporter genes under the control of tissue specific promoters facilitates following iPSC differentiation in vitro, and purification of the desired cell type. This can be achieved by targeting endogenous promoters, or by introducing promoter–

reporter constructs if the promoter region is well characterized and tissue specific. Such an engineered line can be particularly useful when testing new differentiation protocols, because maturation of cells can be screened in a non-invasive and automated fashion. Similarly, for protocols that result in low yield of the desired cell type, activation of fluorescent reporter or antibiotic resistance genes enables purification of rare target cells by FACS or by selection, respectively. Certain reporter genes, such as ferritin or transferrin, permit in vivo tracking of cells using MRI [Gilad et al., 2008], which is particularly useful for non-invasive preclinical animal studies. Additionally, the ability to introduce specific mutations into the gene of interest by gene targeting is particularly valuable in gene function studies. For example, many PD-associated variants are reported in the *PARK2* gene, but for the most part their effects on the function of PARK2 are unknown. Isogenic iPSCs that differ at two PD-associated point mutations in the *SNCA* gene have been recently generated [Soldner et al., 2011]. By introducing individual mutations in the same genetic background, researchers can dissect the precise function(s) of each protein domain and test novel drugs to establish which one has the greatest effect on the given genotype.

Another tempting application of gene targeting by homologous recombination using transcription activator-like effector nucleases (TALENs) or zinc finger nucleases (ZFNs) is the correction of genetic defect in patient's cells, their differentiation into a desired cell type, and autologous transplantation of the corrected cells into the patient. Recent proof of principle experiments demonstrated that a disease related gene can be genetically targeted [Zou et al., 2009], suggesting that it is feasible to correct the genetic defect in a patient's iPSCs.

iPSC derived DA neurons for regenerative medicine. Since the main pathology of PD is caused by a specific loss of A9 DA neurons in the substantia nigra pars compacta, PD is a prime candidate for cell replacement therapy. Induced pluripotent stem cell technology provides opportunity to use patient-derived iPSCs as source of autologous cells for cell replacement. However, before PSC-derived replacement cells can be transplanted into patients, several criterions must be addressed. These include: (1) the derivation of integration-free iPSCs; (2) the differentiation of iPSCs into DA neurons in chemically defined conditions; (3) the scalability of the process enabling reliable manufacturing of the final product in large quantities; (4) the transfer of laboratory developed technology to a good manufacturing practice (GMP) facility; and (5) safety and efficacy of GMP manufactured cells.

Use of integrating viruses as carriers for reprogramming genes poses a potential risk, since even low viral expression may affect differentiation [Yu et al., 2007] and cause malignancies in animal models [Okita et al., 2007]. Soldner and colleagues [Soldner et al., 2009] used excisable viruses to generate factor-free PD patient-specific human iPSCs. Completely zero-footprint and viral-free reprogramming has been achieved using small molecules, espisomal vectors [Yu et al., 2009], recombinant proteins [Cho et al., 2010], and RNA [Warren et al., 2010]. However, these approaches result in iPSC production with even lower efficiency than conventional methods using integrating viruses. The use of Sendai virus as a vector for delivery of reprogramming factors might circumvent the problem of low efficiency of reprogramming with non-integrating vectors [Fusaki et al., 2009]. Sendai virus genome is a negative sense, single-stranded RNA molecule that replicates exclusively in the cytoplasm and enables ample protein synthesis. Furthermore, multiple viral genes carry temperature-sensitive mutations that facilitate the elimination of vector traces in reprogrammed cells. Sendai virus provides efficient delivery and abundant production of reprogramming proteins without the risk of integration into the host genome.

We and others have developed protocols for derivation, maintenance, and differentiation of NSCs and further differentiation into DA neurons in xeno-free chemically defined conditions [Swistowski et al., 2010]. Our protocol for generating transplantable DA neurons is a step-wise process consisting of the following: (1) generation of NSCs (corresponding to neural induction in vivo); (2) differentiation into midbrain DA precursors (midbrain patterning); and (3) maturation into DA neurons (terminal differentiation). Our data demonstrates that each step can be readily accomplished in xeno-free defined conditions. This is important for the potential use of cells for clinical applications where it is essential that cells are produced in a GMP facility using robust, scalable protocols and avoiding any animal products during the manufacturing process. In addition, cells at the intermediate stages of differentiation can be cryopreserved, making the process of production of functional DA neurons from human PSCs scalable. We are in the process of transferring this methodology to GMP facilities for production of clinical-grade functional DA neurons for potential therapeutic applications.

As we discussed earlier, DA neurons were efficiently generated from iPSCs using protocols previously developed for human ESCs [Swistowski et al., 2010]. Importantly, there were no observable differences in neural and DA differentiation between ESCs and iPSCs. Moreover, functional recovery in a PD animal model (6-hydroxydopamine lesioned rats) was detected after the transplantation of iPSC-derived DA neurons into rat brains, similar to results with grafts of human ESC-derived DA neurons in the same PD model [Swistowski et al., 2010].

The next step toward clinical application of iPSC is the derivation of viral- and integration-free iPSC lines under xeno-free conditions, as well as their differentiation under defined xeno-free conditions. Recently, protein-based human iPSCs have been produced and differentiated into DA neurons [Rhee et al., 2011]. Furthermore, Ross et al. [2010] derived human iPSCs in a xeno-free culture system, and DA neurons were successfully produced from iPSCs under xeno-free defined conditions, opening doors toward clinical applications.

CONCLUSIONS

The recent progress in understanding the developmental biology of midbrain DA neurons paved the road toward numerous applications of PSCs for the advancement of the regenerative medicine, disease modeling, and drug discovery. PSCs are a self-renewable source of cells that can be expanded, genetically modified, and differentiated into highly specialized cells, such as DA neurons. Nevertheless, multiple questions and obstacles endure. To begin with, tumorigenic potential of iPSCs derived by integrating approaches raises significant safety concerns and demands improvement of iPSC

derivation methods. The existence of residual epigenetic memory in iPSCs [Kim et al., 2010] necessitates the need to define the best tissue as a source of starting material for reprogramming, depending on the downstream application. Surprising findings that iPSCs induce an immune response following autologous transplantation in mice [Zhao et al., 2011] requires evaluation of the therapeutically valuable cells derived from patient-specific iPSCs before autologous transplantation in humans. Finally, detection of de novo mutations in iPSCs raises additional safety concerns and needs to be further addressed [Gore et al., 2011].

Further refinement of differentiation protocols is needed in order to achieve a higher percentage of DA neurons and reduce contamination with non-DA neuron cells in the final cell product. An issue with disease modeling using patient-specific iPSCs and DA neurons is the identification of measurable phenotypes that can be used as therapeutic targets. This is particularly challenging in cases of complex diseases such as PD, since genetic, environmental, and epigenetic factors play a role. iPSCs may faithfully transfer the genetic component, but the epigenetic and environmental milieu may be more difficult to reproduce and remains a challenge. Finally, there is a demand for improved delivery methods in therapeutic applications of in vitro generated DA neurons. Given the fast pace of iPSC research, we are certainly bound for the exciting ride toward future discoveries.

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