

## Will New Cellular Models of Parkinson's Disease Advance Understanding of Disease and Therapy?

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### Abstract

Virtually all experimental models aimed at mimicking Parkinson's disease (PD) are limited in their ability to replicate clinical and pathological disease phenotypes. A great hope relies on a new technique called 'nuclear reprogramming', to generate cellular models based on the ability to manipulate somatic cells and modify their epigenetic signature, thus producing embryonic stem cell-like cells. This technique is a major advance towards the development of patient-specific cell culture systems, in which PD-specific phenotypes can be studied. These derived embryonic stem cell-like cells are termed induced pluripotent stem cells (iPSCs). Patient-specific iPSC lines lay the foundation for further differentiation into the tissue type of interest for PD, midbrain dopaminergic neurons, but also other cell types of interest and provide a method of creating cells with the specific genetic make-up of a patient. Currently, much effort is focused on refining nuclear reprogramming through factor-free derivation, and further experimental approaches to try to maximise the potential of iPSCs, such as tissue engineering and human-animal chimeras. Overall, iPSC technology is believed to greatly advance three main areas: modelling human disease in a transformative way, providing valuable tools for drug screening approaches and ultimately fulfilling the hope of successful and safe cell-replacement therapies to fight human disease.

### Keywords

Parkinson's disease, experimental models, nuclear programming, induced pluripotent stem cells

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To date, a model for Parkinson's disease (PD) that accurately replicates key features of pathophysiology represents a major bottleneck in the understanding of disease mechanisms, as well as in efficient drug screening. Both cell culture systems and animal models of PD have fundamental limitations in that none of these models faithfully recapitulates all of the clinical and pathological phenotypes that define the disease. Effectively modelling disease progression, with reproduction of pathological hallmarks of the disease, such as Lewy bodies, poses significant challenges to the development of an animal or cellular model of PD.

### Human Cellular Models of Parkinson's Disease

Several different human cellular models have been applied for the study of PD.<sup>1</sup> These include primary patient-specific cell lines, such as fibroblasts, lymphoblastoid cell lines and cybrid cell lines. Other cellular models for investigating PD pathology are human neuroblastoma or carcinoma cell lines (for example, SH-SY5Y, SK-N-SH MN9D and Ntera-2), immortalised human mesencephalic cell lines (for example, Lund human mesencephalic [LUHMES], NGC-407 and ReNcell VM NSC) and stem cells (including those of embryonic, neural [adult and embryonic] and mesenchymal [bone marrow, umbilical] origin). *Table 1* outlines some advantages and disadvantages of the different human cellular

models.<sup>2</sup> Although tissue culture models of disease mechanisms have their experimental limitations, they have some significant advantages over animal models of disease, as they can be human genome-based and allow for the assessment of pathological characteristics in a more time- and labour-efficient manner.

### Reprogramming of Adult Somatic Cells into Induced Pluripotent Stem Cells

Recent discoveries in the field of stem cell biology have led to the ability to reprogramme somatic cells to a pluripotent state in mouse and human models; these cells are termed induced pluripotent stem cells (iPSCs).<sup>3,4</sup> This new approach allows the derivation of patient-specific cell lines from individuals with specific diseases. Cellular reprogramming and iPSCs were hailed as a breakthrough in 2008 and raised hopes that these cell lines may offer the opportunity to elucidate disease mechanisms as well as cure diseases such as PD.

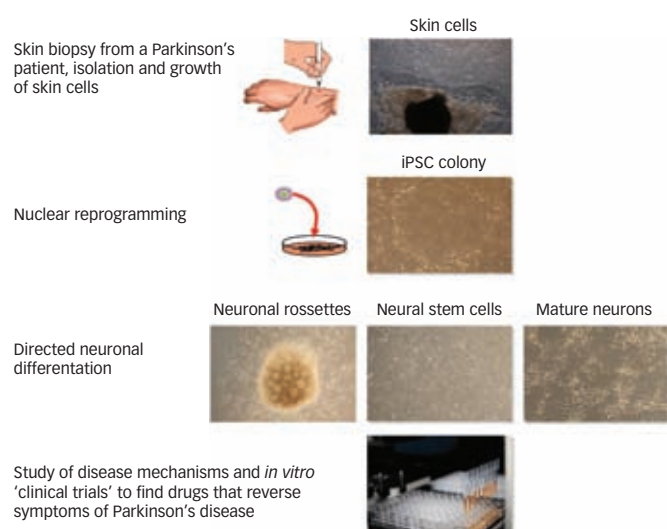
Besides this novel technique of nuclear reprogramming using exogenous transcription factors, there are two other approaches that can successfully be used for nuclear reprogramming: somatic cell nuclear transfer or cloning and cell fusion approaches.<sup>5</sup> Traditionally, cloning involves combining an enucleated oocyte with the nucleus

**Table 1: Advantages and Disadvantages of Human Cellular Models of Parkinson's Disease<sup>2</sup>**

Cell Type	Advantages	Disadvantages
Immortalised or cancer cell lines (e.g. SH-SY5Y, EBV-transformed lymphocytes, LUHMES)	<ul style="list-style-type: none"> <li>• Low cost for maintenance</li> <li>• Homogenous cell population</li> </ul>	<ul style="list-style-type: none"> <li>• Lack important aspects of native function (e.g. metabolic function and electrical properties)</li> </ul>
Primary cell lines (e.g. fibroblasts, cybrid cells)	<ul style="list-style-type: none"> <li>• Fully differentiated cell types</li> <li>• Close approximation of native function, but not necessarily the tissue type affected</li> </ul>	<ul style="list-style-type: none"> <li>• Not easily accessible or available for all cell types</li> <li>• Questionable reproducibility</li> </ul>
Stem cells and induced pluripotent stem cells	<ul style="list-style-type: none"> <li>• High quality</li> <li>• Available source of all cell types</li> <li>• Close approximation of native function</li> </ul>	<ul style="list-style-type: none"> <li>• Maintenance is expensive</li> <li>• Time-consuming to obtain fully differentiated cell types</li> <li>• Require more effort to achieve purified population</li> </ul>

EBV = Epstein-Barr virus; LUHMES = Lund human mesencephalic.

**Figure 1: Modelling 'Parkinson's Disease in a Dish'**



iPSC = induced pluripotent stem cell.

of a somatic cell, which can form an entire organism. This was successfully demonstrated by cloning of the first mammal, Dolly the sheep, in 1997.<sup>6</sup> Embryonic stem cells from non-human primates derived through cloning was first accomplished a decade later in 2007.<sup>7</sup> In contrast, cell fusion, e.g. heterokaryons of mouse embryonic stem cells and human fibroblasts, is another notable technique for reprogramming that is specifically useful for understanding the regulatory programmes that are involved in this process.<sup>8-10</sup>

Transcription factor-based nuclear reprogramming relies on the introduction of exogenous factors into somatic cells that reprogramme or 'rejuvenate' via modification of their epigenetic signature to a state almost indistinguishable from embryonic stem cells. Nuclear reprogramming has been shown to be successful for a variety of adult somatic tissues from all three germ cells, including fibroblasts after more than 20 passages,<sup>11</sup> gingival cells,<sup>12</sup> keratinocytes,<sup>13</sup> hepatocytes,<sup>14</sup> gut mesentery-derived cells,<sup>15</sup> peripheral blood cells<sup>16-18</sup> and amniocytes.<sup>19,20</sup>

Using this technique, a model for 'PD in a dish' can be developed (see *Figure 1*). In simple terms, skin cells from a patient with PD are isolated and expanded in culture for eight to 10 weeks. Depending on the protocol, a combination of specific transcription factors (for example Oct4, Sox2, Klf4 and c-Myc) is introduced into these patient-specific cells, which are then cultured under stem cell

conditions for two to six weeks until stem cell-like colonies appear. These are then manually picked and characterised for markers of pluripotency and differentiation potential.<sup>21</sup>

Transcription factors and genetic delivery methods that are used for the induction of iPSCs may vary, but certain key regulators such as Oct4 cannot be substituted. Initial nuclear reprogramming strategies utilised viral vectors with the caveat that they either do not silence completely or reactivate at a later state, thus hampering the potential to differentiate in specific tissue types. The addition of transcription factors also has the theoretical risk of producing cancer cells. To address this, several factor-free strategies have been proposed for the purpose of nuclear reprogramming, including excision of the reprogramming vector, use of proteins or small molecules (non-DNA strategy), or vectors that do not integrate into the host genome (non-integration strategy).<sup>22,23</sup>

### Mechanism of Nuclear Reprogramming

iPSCs are very similar in their expression pattern to embryonic stem cells. They express pluripotency genes at high levels and lineage-specific genes are suppressed. Over multiple passages, the expression signature of iPSCs become even more similar to embryonic stem cells.<sup>24</sup> Nuclear reprogramming requires a dramatic alteration of the epigenetic landscape<sup>25</sup> and only a few mouse iPSC clones are completely reprogrammed and suitable for producing a completely iPSC-derived offspring, apparently due to a single imprinted gene cluster Dlk1-Dio3.<sup>26</sup> One of the key epigenetic regulators is the activation-induced cytidine deaminase (AID) which acts as a DNA demethylase and 'aids' nuclear reprogramming, as shown in very elegant cell fusion experiments of human fibroblasts and mouse embryonic stem cells.<sup>8</sup> Another epigenetic mark that is crucial for gene activation/silencing is histone modification. Reprogrammed iPSCs re-acquire bivalent marks of H3K4 and H3K27 trimethylation, as in embryonic stem cells, thus reactivating expression of pluripotent genes.<sup>27,28</sup> The completion of nuclear reprogramming is complex, but forward-steps are being made to unravel this astounding process.

### Differentiation into Functional Dopaminergic Neurons

The derived patient-specific iPSCs lay the foundation for differentiation into the tissue-type of interest, i.e. mid-brain dopaminergic neurons that are specifically vulnerable and subject to neurodegeneration in PD. A large body of literature is accumulating on protocols that differentiate embryonic stem cells, or more recently iPSCs, into dopaminergic neurons. The driving force behind this

research has been to engineer cells suitable for cell replacement therapy. However, the value of the system for the study of disease pathways for PD and drug discovery has now gained a lot of interest.

In general, culture conditions for directed differentiation into dopaminergic neurons have been developed to mimic the microenvironment present during embryogenesis, using specific signalling factors or genetic engineering.<sup>29-36</sup> Recently, a series of elegant studies has elucidated the molecular code for the generation of midbrain dopaminergic neurons; these require the temporal and spatial expression of specific neurotrophic and signalling factors.<sup>37-39</sup>

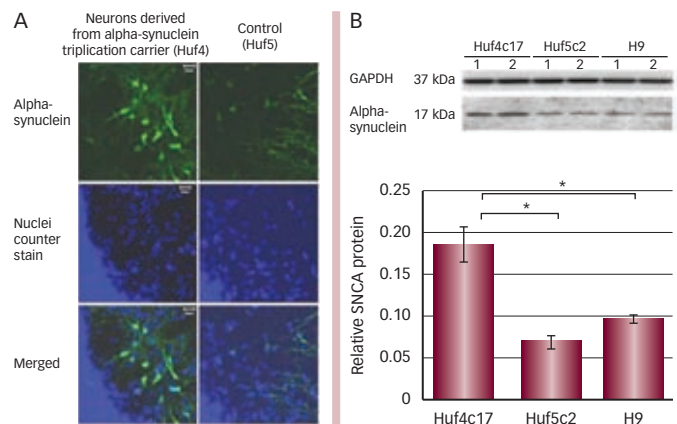
The key challenge for all dopaminergic differentiation protocols is the derivation of high-yield authentic dopaminergic neurons and the complete conversion of undifferentiated stem cells into post-mitotic neurons. Three strategies for deriving dopaminergic neurons have been established: embryoid body-derived neural stem cells further differentiated into dopaminergic neurons,<sup>40</sup> stroma-induced differentiation using PA6 or MS5 feeder cells<sup>33,41</sup> and, more recently, direct differentiation into dopaminergic neurons by inhibition of meso- and endodermal lineage.<sup>42</sup> Neuronal differentiation using the embryoid body technique is described in *Figure 1*. Beginning with the iPSC colony, embryoid bodies are formed over four to eight days in culture. Embryoid bodies are subsequently plated onto special coated plates, which facilitate attachment and form neuronal rosettes ('rose-like' structures). Neuronal rosettes are isolated and cultured as neuronal stem cells. Neuronal stem cells can be passaged and cryopreserved. Finally, combinations of signalling factors and growth factors are introduced to induce their differentiation into dopaminergic neurons.<sup>40,43</sup>

### Parkinson's Disease-specific Induced Pluripotent Stem Cell-derived Cell Lines

To date, there are only a few reports that have generated iPSCs from patients with PD. The main challenge is not to reprogramme patient-specific somatic cells or differentiation into dopaminergic neurons, but to define a specific disease-associated phenotype. In an early publication in 2008, a variety of disease-specific iPSCs were generated including a case with idiopathic PD (57-year-old male, Coriell repository AG20446).<sup>44</sup> Another publication reported the derivation of functional iPSC-derived dopaminergic neurons from five patients with parkinsonism. The fibroblasts were also obtained from the Coriell repository from individuals with an age of onset between 44 and 83 years and parkinsonian symptoms. None of the patients had a known genetic cause of PD.<sup>45</sup> One of the potential reasons for the absence of a phenotypic difference in these iPSC-derived neurons is that these cases had no specific underlying disease-causing mutation and their diagnosis of PD was caused by other factors such as environmental exposure.

We generated iPSC-derived neurons from a patient with one of the most severe genetic forms of PD, genomic triplication of the alpha-synuclein gene (four gene copies in total) presenting a clinically extreme progressive form of PD. Neurons from this patient show alpha-synuclein upregulation in iPSC neurons compared with control cells, using Western blot analysis and immunocytochemistry (see *Figure 2*). This shows that the effect of the mutation is maintained in iPSC-derived mature neurons as alpha-synuclein expression is increased ~2-fold in the cells from this patient (Byers et al., personal

**Figure 2: An Example Of Neuronal Induction Of Induced Pluripotent Stem Cells from a Patient with Parkinson's Disease who is an Alpha-synuclein Triplication Carrier and an Unaffected Sibling**



GAPDH = glyceraldehyde 3-phosphate dehydrogenase; SNCA = alpha-synuclein.

communication). Also of note is that alpha-synuclein expression is increased in the differentiated neurons similar to levels in the adult brain, whereas the original fibroblasts from this patient express very low levels of alpha-synuclein that are not detectable by standard protein blotting techniques (data not shown). These data are promising preliminary findings for further identification of a pathological disease phenotype, i.e. abnormal protein aggregation. With regard to the use of these cells for cell replacement strategies, the publication of Hargus and colleagues is noteworthy.<sup>46</sup> Here, differentiated iPSCs derived by Soldner et al.<sup>45</sup> were transplanted into adult rodent striatum and showed survival as well as a behavioural recovery in the toxicological 6-hydroxydopamine (6-OHDA) animal model. This is an early indication that these cells could be suitable for therapeutic approaches in the future.

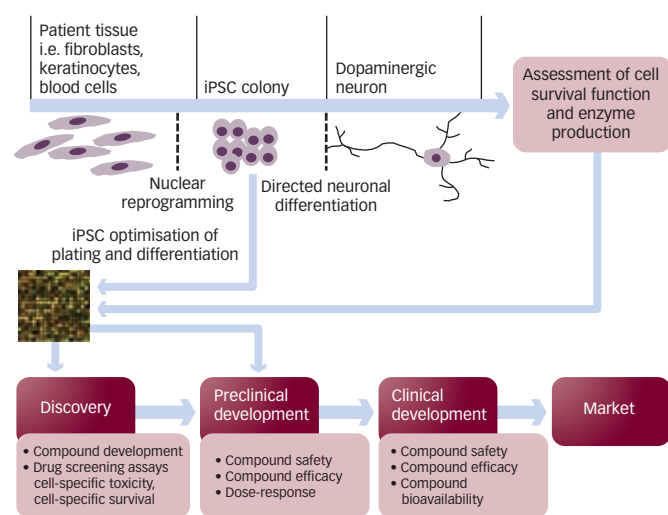
### Drug Discovery Using Patient-specific Induced Pluripotent Stem Cells

As mentioned earlier, high-throughput drug screening is an important potential use of iPSC-derived PD neurons. The great advantage of human iPSCs for drug screening is the biological response for toxicity and drug metabolism, which can be challenging in current animal models. Also, cancer cell lines are used for toxicological testing and presumably due to their tumourigenic potential, behave very differently to the target tissue-type, for which a drug is to be developed. The hope for iPSCs is to reduce the failure rate of compounds and quickly develop effective drugs. A hypothetical process for adapting iPSC neurons for high-throughput screening is depicted in *Figure 3*.<sup>2</sup> For entry into the drug development process, the concluding stage of iPSC preparation requires a 'pure' population of iPSC neurons, miniaturisation assays and novel high-throughput imaging approaches.<sup>47,48</sup> If well-defined cell populations with robust phenotypes can be developed, these cell lines could become a valuable asset for assaying drug libraries and lead compounds can be tested quickly on a human background of iPSCs.<sup>2</sup>

### Challenges of Induced Pluripotent Stem Cells as a Novel Model of Parkinson's Disease

Dopaminergic neurons derived from patient-specific iPSCs represent a promising new model for PD, but challenges remain at several levels. At the level of nuclear reprogramming, problems include the

**Figure 3: Future Development: Adaptation Of Induced Pluripotent Stem Cells for High-throughput Screening?**



iPSC = induced pluripotent stem cell.

generally low efficiency of the process (0.01–0.1% of the somatic cells become iPSCs) and the ineffective silencing/reactivation or excision of transgenes, which can prove problematic for further differentiation of these cells and which will hopefully be overcome by chemical induction of pluripotent stem cells.

The next level of complexity is the neuronal differentiation into functional mid-brain-specific dopaminergic neurons of the A9 region. Most cultures still contain very heterogeneous cell populations of undifferentiated and neural precursor cells, and optimised protocols are warranted to reproducibly differentiate these into high-yield dopaminergic neurons with correct regional specification.

Another challenge as discussed previously, is the PD-specific phenotype in iPSC-derived neurons. One reason for not seeing a

phenotype could be that the dopaminergic neurons are 'young' and do not represent 70-year-old neurons in the brain of a patient. One approach could be to promote ageing in a culture dish by introducing or silencing genes that are known to accelerate ageing or to transplant these neurons into an animal and study the transplanted cells in the context of a functional organism.

## Perspectives and Advances in Cellular Models

There is a great excitement and effort within the PD community to derive iPSCs from patients with PD to model disease, use the cells for drug discovery and ultimately have approaches for cell transplantation.

Even though this new technology is in its infancy, there are already efforts under way to advance the current models of iPSCs. The generation of isogenic panels of iPSCs using gene modification or correction approaches (zinc-finger technology or viral methods) to directly study the effect of presumably causative mutations. These iPSCs are theoretically only different in respect of the disease-causing mutation, thus representing a 'genetically virtually identical' control cell line.<sup>49,50</sup> Another approach to increase the efficiency of neuronal differentiation is the engineering of iPSCs that can express mid-brain-specific transcription factors with the goal of more specific mid-brain dopaminergic differentiation, but forced expression of transcription factors could also lead to 'incomplete' phenotypes.<sup>51</sup>

## Summary and Conclusions

In summary, patient-specific iPSC-derived neurons can be generated *in vitro*, but further 'fine-tuning' of the model is needed to reproduce a pathological phenotype. Overall, iPSC technology has the potential to transform the PD scientific arena and is believed to greatly advance three main areas: modelling disease on a human cellular background, providing valuable tools for drug screening approaches and, ultimately, fulfilling the hope of successful and safe cell-replacement therapies to fight human disease. Thus, patient-specific iPSCs show great promise for significant advances in the treatment of PD. ■

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