Cellular reprogramming: a new approach to modelling Parkinson's disease

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Abstract

iPSCs (induced pluripotent stem cells) offer an unparalleled opportunity to generate and study physiologically relevant cell types in culture. iPSCs can be generated by reprogramming almost any somatic cell type using pluripotency factors such as Oct4, SOX2, Nanog and Klf4. By reprogramming cells from patients carrying disease-associated mutations, and subsequent differentiation into the cell type of interest, researchers now have the opportunity to study disease-specific cell types which were previously inaccessible. In the case of PD (Parkinson's disease), reprogramming is advancing rapidly, and cell lines have been generated from patients carrying mutations in several disease-associated genes, including *SNCA* (α-synuclein), *PARK2* (parkin), *PINK1* (phosphatase and tensin homologue deleted on chromosome 10-induced putative kinase 1), *PARK7* (*DJ-1*) and *LRRK2* (leucine-rich repeat kinase 2), as well as idiopathic cases. Functional dopaminergic neurons have been differentiated from these cells and their physiology has been compared with control neurons. Human dopaminergic neurons had been previously inaccessible until post-mortem, when the disease is generally highly progressed into pathology. In comparison, iPSCs provide a living cell model with the potential to study early molecular changes which accumulate in cells and ultimately result in neurodegeneration. Although clear phenotypes have not yet been unambiguously identified in patient-derived dopaminergic neurons, there are suggested aberrations in cellular pathways involved in neurodegeneration. Overall, these cells offer a unique opportunity to study dopaminergic neurons carrying a 'Parkinsonian genome'. The present review discusses the advances in cellular reprogramming technologies and studies that have been carried out on PD-derived iPSCs and differentiated dopaminergic neurons.

Parkinson's disease

PD (Parkinson's disease) is the second most common neurodegenerative condition in the Western world and its frequency is set to increase as our life expectancy continues to rise. PD is a progressive condition which is characterized by the loss of dopaminergic neurons from the substantia nigra pars compacta, ultimately resulting in the presentation of motor and non-motor symptoms that characterize this disease. Post-mortem analysis of PD brains reveals the accumulation of protein aggregates (Lewy bodies), but it is unclear whether these inclusions are a consequence or a cause of the pathology of PD. The origins of this complex disease remain elusive and, although several genes have been linked with familial forms of PD [*SNCA* (α-synuclein), *PARK2* (parkin), *PINK1* (phosphatase and tensin homologue deleted on chromosome 10-induced putative kinase 1), *PARK7* (*DJ-1*), *LRRK2* (leucine-rich repeat kinase 2) and *GBA* (acid β-glucosidase)] [1–3], the vast majority of cases appear to be idiopathic, which probably reflects the complex interaction between genetic susceptibility and environmental factors. It is the combined impact of genes and environment which makes modelling PD so challenging.

Mutations in the *LRRK2* gene are found in both sporadic (1 % of all cases) and familial (4 % of cases) forms of PD and cases are indistinguishable from sporadic PD in the clinic [4]. The *LRRK2* gene encodes a large multidomain protein, the functions of which remain largely unknown. Point mutations identified in PD cluster around three major domains of the protein: the ROC (Ras of complex proteins) GTPase domain, the COR (C-terminal of ROC) domain and the kinase domain. Several studies in cell models have demonstrated that the G2019S mutation increases kinase activity and that this may mediate dopaminergic neuron degeneration [5,6]. Mutations outwith the kinase domain have also been linked to increased kinase activity [7] and the GTPase domain has been shown to regulate the activity of the kinase domain [8]. Therefore mutations in either of these regions are likely to alter physiological function.

Key words: induced pluripotent stem cell (iPSC), leucine-rich repeat kinase 2 (LRRK2), Parkinson's disease, reprogramming.

Abbreviations used: iPSC, induced pluripotent stem cell; LRRK2, leucine-rich repeat kinase 2; LTR, long terminal repeat; 6-OHDA, 6-hydroxydopamine; PD, Parkinson's disease; PINK1, phosphatase and tensin homologue deleted on chromosome 10-induced putative kinase 1; ROC, Ras of complex proteins; *SNCA*, α-synuclein.

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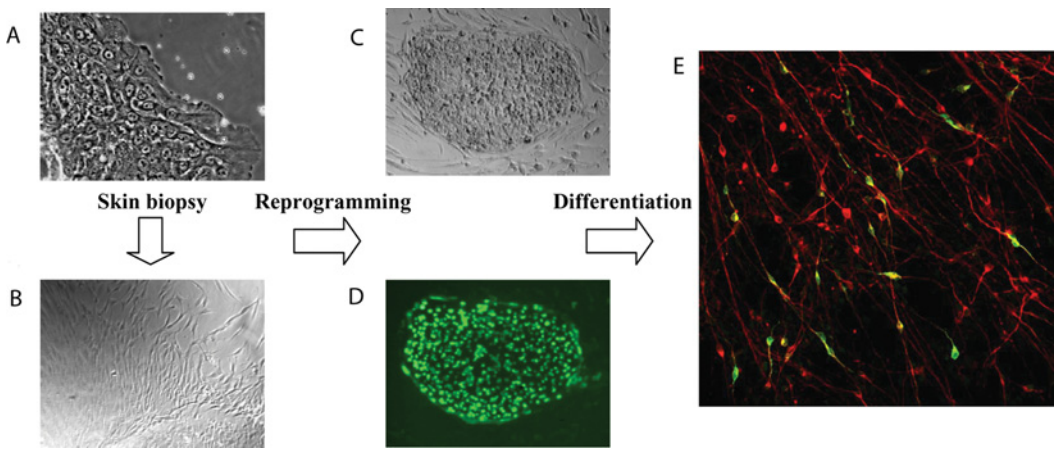
Current models of PD

Many different cell and animal models have been developed in an attempt to recapitulate the neurodegenerative and motor phenotypes of PD. Early animal models of PD relied on the administration of neurotoxins that target dopaminergic

Figure 1 | **Reprogramming and differentiation of PD patient-specific iPSCs**

Schematic representation of iPSC reprogramming workflow. A small biopsy of somatic cells is taken from patients and/or controls by skin punch (**A**) and fibroblast cells grow out from the biopsy sample *in vitro* (**B**). A combination of reprogramming factors is delivered to these cells, typically by viral transduction, and cells are maintained in culture until stem cell colonies appear (**C**). Pluripotency can be assessed by a number of markers, including live cell staining for Tra-1-81 (**D**), which confirms the cells are iPSCs. The iPSCs can then be differentiated into midbrain dopaminergic neurons (**E**). (**E**) Dopaminergic neurons differentiated from an idiopathic PD patient stained by immunocytochemistry (Tuj1, red; tyrosine hydroxylase, green).



neurons via the DAT (dopamine active transporter); for example, administration of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [9] or 6-OHDA (6-hydroxydopamine) produces motor deficits which mimic those observed in human PD. Although understanding the pathways resulting in dopaminergic neuron death in such models may be applicable to PD, these models do not faithfully recapitulate the slow progression of neurodegeneration observed in human patients and do not take into account the effect of genetic mutations. Knockout and transgenic mouse models have been used extensively in PD research, as have alternative non-mammalian systems, such as *Drosophila*, *Caenorhabditis elegans* and zebrafish [10], some of which show motor deficits and neurodegeneration. However, the lack of brain structure complexity or short lifespan of these models can mean that comparisons with human disease are difficult to interpret. Overall, animal models have been useful for elucidating disease mechanisms and providing insight into gene function. However, it is likely that modelling PD in the very same human neurons which die in the disease will be necessary to discover the therapies of the future which will actually prevent neurodegeneration rather than provide only symptomatic relief as is currently the case in PD.

In the present paper, we describe how more relevant human cellular models can be obtained by utilizing reprogramming technologies which can convert somatic cells into either iPSCs (induced pluripotent stem cells) or directly into other somatic cell types. After reprogramming, iPSCs from PD patients can be differentiated into midbrain dopaminergic neurons to sudy their physiology *in vitro.* These cells are advantageous from two perspectives. First, they are a source of human midbrain dopaminergic neurons that are otherwise unobtainable. Post-mortem data provide an insight into the pathology of the brain; however, this is at the end stage of PD, from which it is difficult to elucidate the early molecular mechanisms of the disease. In order to develop new therapies to prevent neurodegeneration, it will be essential to identify the early changes in cellular physiology which eventually lead to pathology. Secondly, patient-derived iPSCs will carry the genetic background of the disease and may help to uncover different genetic interactions which may lead to PD. Taken together, reprogramming technologies offer a unique opportunity to study PD disease mechanisms using cells that have derived directly from patients (Figure 1).

Reprogramming technologies

The first reprogramming experiments were carried out by Takahashi and Yamanaka [11] in mouse fibroblasts and completely revolutionized the stem cell field. They used retroviruses to deliver transcription factors (Oct4, Sox2, c-Myc and Klf4) that confer pluripotency on somatic cells, resulting in transformation from differentiated fibroblasts into teratoma-forming cells termed iPSCs. Shortly after this seminal work was published, the Yamanaka [12,13] and Thomson [13a] laboratories succeeded in reprogramming human fibroblast cells using Oct4, Sox2, c-Myc and Klf4, or Oct4, Sox2, Nanog and Lin28 respectively. These factors have been shown to be sufficient to induce conversion of somatic cells into iPSCs, although concerns have been raised about using oncogenes in the reprogramming process, particularly if iPSCs may become a source of stem cells for transplantation. Although the exogenous transcription factors have been shown to be silenced during the reprogramming process,

there is still some concern over their reactivation, particularly for c-*Myc* and *Klf4*, which have been linked to oncogenesis. Mice generated directly from reprogrammed iPSCs have been shown to have a high incidence of tumorigenesis which was as a direct result of c-Myc reactivation [13]. Hence the use of Nanog and Lin-28 instead may be advantageous.

Following the identification of these minimal sets of reprogramming factors, research has focused on improving reprogramming efficiencies using new methods. The use of integrating retroviral vectors to reprogramme cells has drawbacks, as multiple insertions in random locations in the genome and incomplete silencing of exogenous genes has raised concerns over the use of iPSC-derived cells for therapeutic purposes. Despite the wealth of information that can be obtained from these cells regarding disease mechanisms and drug development, unregulated expression of exogenous factors could alter cellular behaviour, thus distorting the results. Two elegant reprogramming systems have been described whereby the virally delivered genes integrate briefly during the conversion process and are later excised, leaving the iPSCs free from vector sequences. The first system utilized lentiviral vectors with a Cre-loxP site in the 3′ LTR (long terminal repeat), which is duplicated into the 5′ LTR upon genomic integration. Once iPS colonies appeared, Cre recombinase was introduced by transient transfection and the viral sequences were excised [14]. The second system is termed the *piggyBac* system and uses transposons to deliver the reprogramming factors. Again, once iPSC colonies had appeared, a plasmid expressing transposase was introduced into cells which induced excision of the exogenous factors [15]. More recently, non-integrating strategies have been developed, including adenoviral vectors [16], repeated transfection of plasmid DNA [17,18], RNA [19,20] or miRNA (microRNA) [21] and protein transduction [22,23]. Whereas non-integrative strategies improve safety with regard to oncogenesis, these methods are generally less efficient at cellular reprogramming. Retroviral delivery of the original Yamanaka factors remains to date the most reliable and robust method available for routine iPSC generation.

Further advances have been made in reprogramming technologies, in which adult cells can be directly converted from one cell type into another, even across different lineages. For example, functional neurons have been generated directly from adult fibroblasts via expression of a minimal set of factors [*ASCL1*, *BRN2* (also called *POU3f2*) and *MYTL1*] which converted adult mouse fibroblast cells into neurons [24]. This has been refined further and, more specifically, functional dopaminergic neurons have been directly converted from human fibroblasts. Two groups defined different minimal conversion sets that resulted in the generation of dopaminergic neurons: Ascl1, Nurr1 and LMX1a [25] and Ascl1, Brn2 and Myt1l were shown to be sufficient to induce neurons and, when used in combination with FoxA2 and Lmx1a, dopaminergic neurons could be generated [26]. Direct reprogramming strategies could eliminate the need for the intermediate iPSC step, but the yields are very low, and multiple fibroblast samples would need to be taken from patients in order to generate large numbers of neurons.

Although it has been shown that PD fibroblasts can be directly converted into dopaminergic neurons [25], this work is still in its infancy, and further tests will be needed to confirm the karyotype of these cells, to ask whether cell fate is stable after withdrawal of reprogramming factors, and to determine whether the cells have been completely reprogrammed.

Reprogramming patient-derived cells

iPSCs are advantageous in that they generate a population of renewable cells that can be directed to differentiate into different cell types. In the field of PD, several iPSC lines have been generated from patient fibroblasts of patients carrying several known PD-related mutations, namely *SNCA* [27,28], *PINK1* [29], *PARK2* [30] and *LRRK2* [31,32] (Table 1). Importantly, these authors report no significant difference in differentiation potential in cells derived from PD patients and controls, therefore they are able to make a fair comparison of neuronal cell biology between different cell lines. Expression of midbrain dopaminergic neuron markers has been confirmed in these cultures, and gene expression arrays have demonstrated that gene expression patterns of differentiated cell populations cluster with rodent midbrain neurons, rather than embryonic stem cells or parental fibroblast cultures [27,28,31,33,34]. Additional functional studies confirmed the dopaminergic nature of these neurons by demonstrating electrical activity by electrophysiology and the production and release of dopamine [25,30,31,35]. More detailed phenotypic analyses on these cells have highlighted some interesting possible gene-specific alterations which may further our understanding of the development and progression of PD. Accumulation of α-synuclein in Lewy bodies in neurons is a common pathological feature of PD, and, as expected in neuronal cultures derived from *SNCA* triplication patients, the total protein levels of α-synuclein were elevated, although this was not conclusively correlated with increased cytoplasmic accumulation in either study [27,28]. Interestingly, higher total levels of α-synuclein were also reported in both studies using LRRK2 iPSC-derived dopaminergic neurons [31,32], whereas no change was reported for the parkin study [30]. It will be interesting to correlate patient iPSC and post-mortem data, as it may be the case that the cell culture system does not mimic the conditions in the brain and that the cells are not aged enough to show abnormal protein aggregation.

LRRK2 patient-derived iPSCs

Some distinct phenotypes have been described in cells derived from LRRK2 patients carrying G2019S mutations. Nguyen et al. [31] have shown that, in addition to increased levels of α-synuclein, LRRK2 cells also expressed higher levels of oxidative stress response genes. By looking into caspase

Table 1 | **PD patient-derived dopaminergic neurons**

Details of studies completed using human dopaminergic neurons differentiated from iPSCs derived from PD patients. The different mutations present in these cells and their phenotypes are described.

Mutation	Reprogramming strategy	Number of patients used in the study	Phenotype	α-Synuclein levels
LRRK2 (G2019S) [31]	Retrovirus: OCT4, SOX2, Klf4	Two lines from one patient, one control and H9	Increased expression of oxidative stress genes; increased susceptibility to H_2O_2, 6-OHDA and proteosome inhibition	Increased total protein levels
LRRK2 (G2019S) [32]	Retrovirus: OCT4, SOX2, Klf4	Four G2019S patients, seven idiopathic patients and four controls. Unclear how many lines used in study	Disturbance in autophagic flux; accumulation of autophagic vacuoles in both idiopathic and G2019S patients	Barely detectable levels in idiopathic and control cells; elevated cytoplasmic levels in G2019S
PINK1 [29]	Retrovirus: OCT4, SOX2, c-Myc and KLF4	Two C1366T patients, one T509G patient and one control. One line per patient used	An 80 % decrease in PINK1 expression; impaired recruitment of parkin to depolarized mitochondrial membranes; reduced clearance of damaged mitochondria and increased levels of mitochondrial biogenesis	Not determined
PARK2 [30]	Retrovirus: OCT4, SOX2, Klf4, c-Myc and Nanog	One patient with heterozygous deletion of exon 3 and 5, one patient with homozygous deletion of exon 3 and two controls	Loss of parkin protein expression; increased spontaneous and evoked dopamine release; decreased dopamine uptake and DAT (dopamine active transport)-binding sites; increased oxidative stress; *MAO* (monoamine oxidase) RNA levels decreased	No difference
SNCA triplication [28]	Retrovirus: OCT4, SOX2, Klf4 and c-Myc	Thirty *SNCA* triplication patients and ten controls	Double α-synuclein RNA and protein levels; increased α-synuclein excreted from cells	Increased RNA and protein levels
SNCA triplication [27]	Retrovirus: OCT4, SOX2, Klf4 and c-Myc	One *SNCA* triplication and one control and H9; three lines from each donor used	Increased expression of protein aggregation and cell death genes; increased susceptibility to oxidative stress	Increased RNA and protein levels

3 activation and cell death, they showed that LRRK2-derived neurons were more vulnerable to several stress agents, including H_2O_2, 6-OHDA and the proteasome inhibitor MG-132. A more recent study [32] using LRRK2 and sporadic PD human iPSC-derived neurons has shown a reduced number of neurites and neurite arborization. In addition, the authors show cytoplasmic α-synuclein in LRRK2 but not control cells, something that we have not observed in our laboratory (E. Hartfield, unpublished work). An accumulation of autophagic vacuoles and disruption of autophagic flux was also reported. Since PD is linked with accumulation of misfolded proteins, defects in the autophagic pathway may contribute to protein aggregation and accumulation of damaged mitochondria, thus increasing levels of oxidative stress in these neurons and making them more susceptible to insult.

Data from LRRK2 patient iPSC-derived dopaminergic neurons remain scarce. However, since much effort has been placed on efficiently reprogramming different cell types and inducing robust midbrain dopaminergic neuron differentiation, efforts can now focus on utilizing these human dopaminergic neurons with relevant PD genomes to elucidate the molecular mechanisms of degeneration. Elegant gene correction technologies can be used to specifically understand gene mutations in PD neurons and to what extent they contribute to the cellular phenotype presented. Soldner et al. [36] have utilized zinc finger nuclease technologies to correct the A53T-coding mutation in the *SNCA* gene in patient-derived iPSCs, adding further value to the use of iPSC-derived dopaminergic neurons as a model for studying of PD pathology.

Limitations of iPSCs

The potential for using iPSCs is immense, but some limitations remain. Differentiation protocols yield mixed neuronal populations, although one can argue that a mixed population containing differentiated dopaminergic neurons, other neuronal types and glia is required as the brain itself is a mixed population. However, care should be taken when analysing these heterogeneous cultures if the goal is to obtain specific midbrain dopaminergic neuron-related phenotypes. One of the main drawbacks with the studies carried out so far is clearly the use of a very low number of different cell lines generated from patients and controls. A more systematic approach needs to be employed, using several iPSC clones derived from several individuals with a similar genetic background. This becomes even more pertinent when using iPSCs derived from idiopathic PD. This has in part been accomplished in one of the latest published studies, by using clonal lines from several control, idiopathic and LRRK2 G2019S lines [32]. This same study also demonstrates the vital importance of using idiopathic cases in these studies, as they showed that many of the phenotypes observed in the LRRK2-derived neurons were also observed on the idiopathic cases, which supports the hypothesis that there will be common underlying pathways in PD.

Conclusions

The generation and differentiation of human iPSCs into neuronal cells is a field in fast expansion, not only for PD, but also for other neurodegenerative diseases, including Huntington's disease [37–39], Alzheimer's disease [40,41], spinal muscular atrophy [42,43] and amyotrophic lateral sclerosis [44]. This technology will have a great impact on our understanding of the pathways involved in the development and progression of diseases, by allowing studies within a completely human *in vitro* system, previously unavailable. By providing a valuable model of study, iPSC technology has the potential for the development of important target identification assays and high-throughput drug screening, identification of disease mechanisms and new therapies in PD.

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