Induced pluripotent stem cell (iPSC)-derived dopaminergic models of Parkinson’s disease

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Abstract

iPSCs (induced pluripotent stem cells) are the newest tool used to model PD (Parkinson’s disease). Fibroblasts from patients carrying pathogenic mutations that lead to PD have been reprogrammed into iPSCs, which can subsequently be differentiated into important cell types. Given the characteristic loss of dopaminergic neurons in the substantia nigra pars compacta of PD patients, iPSC-derived midbrain dopaminergic neurons have been generated to investigate pathogenic mechanisms in this important cell type as a means of modelling PD. iPSC-derived cultures studied so far have been made from patients carrying mutations in LRRK2 (leucine-rich repeat kinase 2), PINK1 [PTEN (phosphatase and tensin homologue deleted on chromosome 10)-induced putative kinase 1], PARK2 (encodes parkin) or GBA (β-glucocerebrosidase), in addition to those with SNCA (α-synuclein) multiplication and idiopathic PD. In some cases, isogenic control lines have been created to minimize inherent variability between lines from different individuals. Disruptions in autophagy, mitochondrial function and dopamine biology at the synapse have been described. Future applications for iPSC-derived models of PD beyond modelling include drug testing and the ability to investigate the genetic diversity of PD.

Parkinson’s disease

Despite decades of research, the complex multi-factorial aetiology of PD (Parkinson’s disease) is still not well understood. The disease manifests primarily as a motor disorder, comprising limb rigidity, slowness of movement (bradykinesia) and resting tremor, all of which result from the progressive degeneration of mDA (midbrain dopaminergic) neurons, specifically those of the substantia nigra pars compacta. Post-mortem neuropathological examination of the remaining dopaminergic neurons reveals the presence of aggregates of the presynaptic protein α-synuclein, called Lewy bodies; however, these aggregates are found throughout the brain stem and subsequently, in later stages of PD, in cortical regions [1]. Mutations leading to familial PD have been found in 11 genes out of 16 identified disease loci (http://www.pdgene.org/linkage.asp), notably including dominant mutations in LRRK2 (leucine-rich repeat kinase 2), recessive mutations in PARK2 (encodes parkin) and PINK1 (PTEN [phosphatase and tensin homologue deleted on chromosome 10]-induced putative kinase 1), and both rare dominant mutations and multiplications of the gene encoding α-synuclein (SNCA). However, over 90% of PD cases occur sporadically (idiopathic PD) and are likely to have a mixed aetiology arising from inherited polymorphisms conferring genetic susceptibility, environmental influence and, most notably, age [2]. The clear monogenic forms of PD have been studied using a wealth of animal and cellular models in order to elucidate possible disease mechanisms that could connect to provide a unifying pathway for PD. One new technology is the use of iPSCs (induced pluripotent stem cells) generated from patients themselves. The present review describes the use of iPSC-derived models to study PD.

Generation of iPSCs

During development, all somatic cells are derived from stem cells that progressively differentiate towards an ever more restricted cell fate, locked in by epigenetic marks and reinforced by transcription factors [3]. The Nobel Prize in Physiology or Medicine (2012) was awarded to Shinya Yamanaka and Sir John B. Gurdon for their groundbreaking work on reversing this process in vitro, allowing virtually any terminally differentiated cell to be reprogrammed to a pluripotent state; the iPSC was born. Yamanaka used retroviral delivery of four transcription factors, Oct-3/4, Sox2, Klf4 and c-Myc (later named ‘Yamanaka factors’), to induce pluripotency in fibroblasts from mice [4] and humans [5].

Of greatest relevance to those who study human disease is the ability to reprogram patient fibroblasts into iPSCs and subsequently differentiate them into relevant cell types for...
study, the concept of ‘disease in a dish’ modelling [6]. Naturally the mDA neurons that are critically lost in PD are being widely studied and the results of such studies are summarized later in the present review. Reports have also been made of direct reprogramming, the conversion of fibroblasts into a desired terminal cell type without the need for a pluripotent intermediate; however, although mDA neurons have been generated from patients as a proof of principle [7], these have not yet been phenotyped to model PD.

Clonal variability and genome editing

Variability between iPSC lines is one of the major potential limitations to this technology. Typically several colonies are chosen from each culture of reprogrammed fibroblasts and are subjected to quality-control measures including verification of transgene silencing and karyotype analysis. However, these clones, colonies derived from the same human donor, still exhibit a large variability [8]. Much of this variability may be explained by the different genomic integration sites of the retroviral vectors expressing the reprogramming factors in each clonal line. The original reports of the generation of iPSCs from mouse and human fibroblasts showed that retroviral sequences had made approximately 20 integrations into each clone [4,5], particularly high due to the delivery of factors by individual retroviruses. One outcome of variability between iPSC lines is a differing efficiency to which lines differentiate into neurons [9].

One way to combat variability between PD and control lines has been to use genome editing to introduce point mutations into the genome, resulting in clonal cell lines that are isogenic, differing only at the changed base(s) [10–12]. Furthermore, clonal variability may be minimized by using non-disruptive methods of reprogramming, such as Cre-exciscible viruses [13], EBNA-1 (Epstein–Barr nuclear antigen 1)-based episomal plasmids [14] or non-integrating viral vectors, e.g. Sendai virus [15]. The importance of this genomic purity was highlighted by genome-wide gene expression profiling, whereby virus-excised iPSCs were more like embryonic stem cells than like virus-integrated iPSCs [13].

One potential consequence of long-term culture of iPSCs is the generation of chromosomal abnormalities over time. Although each line is subjected to analyses of karyotype, including by analysing the copy number of many single nucleotide polymorphisms, detailed examination reveals small copy number variants in both iPSC and embryonic stem cell lines that may often go undetected [10].

Variability between lines may reduce the ability to perceive meaningful phenotypes, especially when only a few lines are used for study, which has been the case for most reports. As this technology is now established it will be important to verify findings in larger numbers of replicates, including isogenic replicates, or to otherwise minimize variability in order to report valid results.

iPSC models of familial PD: LRRK2, SNCA, PARK2 and PINK1

Our understanding of the molecular basis of PD has increased significantly through the study of inherited familial forms of the disease, as this allows for the direct comparison between models carrying mutations and those without. However, all models, be they animal, cellular or in vitro, are limited in their relevance to PD in some way, either by species or cell-type differences or through overexpression of genetic constructs. iPSC-derived cultures offer a unique advantage in that we are able to study human cells from patients and controls. Furthermore, these cultures can be differentiated into the particular cell type that is inherently vulnerable in PD, mDA neurons, the loss of which gives rise to the major motor symptoms. Certainly iPSC technology provides the opportunity for an unprecedented insight into this human cell type, although the iPSC-derived mDA neurons currently lack a significant population of glia, a physiological network of connections and a mature arborization with a high energy demand [16]. However, mDA neuronal cultures still offer powerful insights into the cell biology of disease in the same cell type that degenerates, allowing us to identify dysfunctional pathways.

LRRK2 and SNCA

The G2019S mutation in LRRK2 is the PD model that has been most studied in iPSC-derived cultures. Multiple reports show that iPSC-derived TH (tyrosine hydroxylase)-positive mDA neurons bearing G2019S exhibit a baseline increase in apoptotic markers, such as cleaved caspase 3 [9,17] and increased susceptibility to apoptosis induced by oxidative stressors [12,17]. This suggests that mutant LRRK2 causes dysfunctionality in cellular pathways within mDA neurons. Two principal aspects of cell biology have been investigated in mutant LRRK2 iPSC-derived cultures: autophagy and mitochondrial function.

Autophagy is the process by which cells degrade damaged proteins and organelles, and is further induced by starvation in order to release needed biomolecules. Macropathology involves the formation of double-membrane-bound autophagosomes within the cytoplasm, engulfing target organelles, before fusing with lysosomes containing degradation enzymes (Figure 1). Autophagosomes can be detected by the presence of LC3 (microtubule-associated protein light chain 3), notably in its conjugated form with phosphatidylethanolamine (LC3-II) [20], and also by specific cargoes such as p62 [9]. iPSC-derived mDA neurons bearing mutant LRRK2 contain increased LC3-positive and p62-positive puncta, and overall the mixed cultures show increased basal LC3-II levels [9]. Blockade of autophagy by lysosomal inhibition showed a mutant-specific reduction in autophagic flux by LC3-II immunoblotting, suggestive of compromised clearance of autophagosomes [9].

Alternatively, proteins may enter the autophagic process directly at the lysosomes, as in CMA (chaperone-mediated autophagy). Here, Hsc70 (heat-shock cognate 70 stress...
Figure 1 | Significant phenotypes in iPSC-derived mDA neuronal cultures used to model PD

Representation of the majority of the phenotypes discussed in the present review that have been verified and/or elucidated in iPSC-derived mDA neuronal cultures bearing mutations in LRRK2, PARK2, PINK1 or GBA. LRRK2 exerts some of these effects from a position of being associated with membranes, notably those of the endocytic-autophagic pathway [18,19]. The process of macroautophagy is detailed in full, showing the formation of a double-membraned autophagosome around damaged organelles, e.g. mitochondria, and subsequent fusion with a lysosome to form an autophagolysosome where degradation will take place. The box in the upper-right-hand corner depicts a presynaptic dopaminergic terminal showing increased spontaneous dopamine release and decreased dopamine re-uptake via the dopamine transporter, α5, α-synuclein.

Mutant LRRK2
1. Deficit in autophagosome clearance
2. Deficit in CMA by inhibition of LAMP2A multimerisation
3. Increased ERK1/2 phosphorylation
4. Increased DRP1 activation
5. Increased susceptibility to mitochondrial damage
6. Increased ROS production

Mutant parkin or PINK1
7. Increased susceptibility to mitochondrial damage
8. Increased ROS production
9. Decreased mitophagy
10. Impaired dopamine synaptic biology

Mutant GBA
11. Deficit in lysosomal activity and autophagy
12. Reduced expression of GBA beyond the endoplasmic reticulum

protein) delivers proteins to LAMP2A (lysosome-associated membrane protein 2A) in the lysosomal membrane where it subsequently forms a protein translocon [21]. iPSC-derived G2019S mDA neurons have accumulation, but not aggregation, of α-synuclein [9,21], but this increase is only at the protein level and not at the mRNA level, suggesting a reduced degradation of α-synuclein protein [12]. Increased co-localization of α-synuclein with LAMP2A puncta in iPSC-derived G2019S mDA neurons, accompanied by non-iPSC mechanistic studies that suggest an inhibition of LAMP2A multimerization by LRRK2, indicates compromised degradation of α-synuclein by CMA [21]. Although both wild-type and mutant LRRK2 inhibit CMA, G2019S LRRK2 is itself more resistant to degradation by CMA and so is able to exert a more inhibitory effect, leading to α-synuclein accumulation [21]. iPSC-derived mDA cultures from SNCA triplication patients show increased expression of SNCA mRNA and α-synuclein protein [8]; this led to increased susceptibility to apoptosis induced by oxidative stressors, but without any steady-state increase in apoptosis [22]. Thus some, but not all, disease mechanisms may be shared between LRRK2 and α-synuclein.

Deficits in autophagy may be the underlying explanation for other described phenotypes. iPSC-derived G2019S mDA neurons show decreased arborization and neurite outgrowth [9,12,23], recapitulating earlier findings in non-iPSC studies linked to autophagy [24]. Furthermore, the same phenotype was induced by overexpression of wild-type or G2019S LRRK2 in control iPSC-derived cultures [9] or rescued by LRRK2 inhibition [12]. mDA neuronal cultures from additional iPSC lines generated by genome editing to either correct or introduce the G2019S mutation further corroborated this mutant phenotype [12]. Studies in non-iPSC models have suggested a connection between LRRK2
and ERK1/2 (extracellular-signal-regulated kinase 1/2) in the induction of autophagy [24,25]. iPSC-derived mDA cultures from isogenic G2019S LRRK2 lines exhibited mutant-specific increased apoptosis and decreased neurite outgrowth, along with alterations in the expression of several genes controlled by pERK (phosphorylated ERK), that could all be rescued by LRRK2 inhibition [12]. Thus pERK may be an important connection between LRRK2, autophagy and apoptosis.

LRRK2 iPSC-derived mDA neurons show shorter more fragmented mitochondria [23]. As a result, cultures exhibit decreased mitochondrial membrane potential and ATP production, increased ROS (reactive oxygen species) [23] and increased sensitivity to toxicity of potassium, but not proton, ionophores, that could be partially rescued with LRRK2 inhibition [26]. Mitochondrial deficits exhibited in iPSC-derived G2019S mDA neuronal cultures could be rescued by inhibition of DRP1 (dynamin 1-like protein) or expression of a dominant-negative DRP1 mutant [23]. Other analysis showed LRRK2 phosphorylation of DRP1, leading to its translocation to mitochondria where it induces mitochondrial fission [23]. In a synthesis of the pathways of autophagy and mitochondrial dynamics, DRP1 manipulation also rescued the mutant-specific increase in lysosome activity and LAMP2 density within iPSC-derived mDA neurons [23].

PARK2 and PINK1

Mutant alleles of PARK2 and PINK1 respectively encoding the E3-ubiquitin ligase parkin and a kinase residing in the mitochondrial membrane, result in recessive PD with early age of onset. Although not the only type of mutation in PARK2, all iPSC lines that have been described are from patients with exon deletions; in the case of PINK1 they are from patients with a mixture of nonsense and missense mutations. PINK1 has been shown to function upstream of parkin, involved in recruiting parkin to damaged mitochondria, such as following mitochondrial depolarization, and it is suggested that mitochondrial parkin initiates the induction of mitophagy (autophagic degradation of mitochondria) [27]. PINK1 mutant iPSC-derived mDA neuronal cultures have more mitochondria, suggesting impairment of mitophagy upon mitochondrial depolarization, when they fail to recruit parkin to mitochondria [28]. Delivery of wild-type PINK1 to these cultures reduced mitochondrial copy number, perhaps by permitting the induction of mitophagy [28]. However, subsequent specific investigation of mitophagy in normal fibroblasts showed that endogenous levels of parkin are insufficient to induce mitophagy in combination with depolarization, requiring parkin overexpression to drive mitophagy [29]. When this was subsequently tested in iPSC-derived mDA neuronal cultures, mitophagy could not be induced by parkin overexpression, even though neurons endogenously express more parkin than fibroblasts [29]. This suggests that mitophagy is not a significant result of mitochondrial depolarization in mDA neurons. However, another group found that control neurons did undergo mitophagy on depolarization, and that it could be blocked by bafilomycin A1, whereas PARK2 mutant neurons lacking functional parkin were unable to induce mitophagy [30]. Further investigation is required here, particularly as each group used different secondary measures to determine whether or not mitophagy had taken place (see also [31] for a more detailed review).

What functions might mutant PINK1 and parkin have in mDA neurons aside from a role in mitophagy? PINK1 mutant iPSC-derived mDA neuronal cultures are more sensitive to cell death and production of ROS elicited by mitochondrial and oxidative stressors, and further showed increased basal oxygen consumption and proton leakage suggestive of intrinsically damaged mitochondria [26]; interestingly, the cellular dysfunction resulting from treatment with valinomycin could be rescued by LRRK2 inhibition [26]. The increased severity of these sensitivities in neurons compared with fibroblasts from the same patients highlights the relevance of using iPSC-derived mDA cultures [26]. PARK2 mutant iPSC-derived neurospheres or neural precursors likewise show increased ROS production [30], including stimulation by manganese toxicity [32], and their mitochondria show abnormal morphology by electron microscopy [30]. Furthermore, parkin has been investigated with respect to dopamine biology, revealing that mutant mDA neuronal cultures have reduced dopamine uptake due to reduced dopamine transporter expression on the cell surface and increased spontaneous dopamine release, both of which could be rescued by expression of wild-type parkin [33]. This is the only report in PD iPSC-derived cultures that has investigated synaptic dysfunction, one of the key areas of PD pathogenesis [2,34]. Synaptic dysfunction requires future attention along with electrophysiological phenotypic descriptions of mature iPSC-derived neurons beyond demonstration of electrical activity.

Overall, the roles for mitochondrial dysfunction and oxidative stress in recessive PD appear to be supported by work in iPSC-derived cultures, but the role of mitophagy in mutant mDA neurons is unclear. As autophagy appears to play a large role in LRRK2 PD, perhaps this reinforces the difference between early-onset recessive PD and the later-onset dominant and idiopathic forms.

Insights into idiopathic PD

Idiopathic PD, accounting for approximately 90% of cases, is likely to be caused by a complex mixture of genetic and environmental risk factors. iPSC lines generated from idiopathic patients lacking known PD mutations offer the opportunity to investigate to what degree patient cells are inherently dysfunctional due to the contribution of a background of genetic susceptibility. Soldner et al. [13] generated the first of these lines, but did not report any phenotypic investigation. The first reported phenotype showed that mDA neurons from idiopathic PD patients did not accumulate α-synuclein, yet did demonstrate the sparser arborization, increased apoptotic markers and autophagic deficits seen in LRRK2 mutant cultures [9]. This
suggests that autophagy deficits are a hallmark characteristic of late-onset PD that converge as the result of either specific monogenic disease or a complex genetic background of susceptibility variants. However, it is worth noting that, although reprogramming generally removes epigenetic marks, those that normally persist through development also persist through the reprogramming process, such as parental imprinting like that seen in Angelman and Prader–Willi syndromes [35].

Homozygous mutations in GBA encoding β-glucocerebrosidase lead to Gaucher’s disease, a severe lysosomal disorder, yet the heterozygous carrier state is a susceptibility variant for PD among idiopathic PD patients [2]. iPSC-derived cultures from patients with Gaucher’s disease show the typical reduction in GBA protein and activity [36–38], yet also demonstrate increased α-synuclein protein in mDA neurons [36] and poor clearance in macrophages [37], supporting the notion that reduced lysosomal GBA has an impact on wider degradation processes, including autophagy. However, studies of the impact of susceptibility variants in GBA on autophagy, as well as a more comprehensive investigation of iPSC-derived cultures generated from idiopathic PD patients, are awaited.

Future implications: genomic diversity and drug testing

Disease models may be used to describe phenotypes that ideally represent disease states, and subsequently are exploited for their ability to screen possible drugs that alter that phenotype. iPSC-derived mDA neuronal cultures generated from PD patients occupy a unique place in the drug screening landscape as they are cells from human patients and one of the critical cell types for their disease. On one level, this technology may be used to screen for drug toxicity in patient cells, including in other differentiated cell types such as hepatocytes [39]. However, perhaps greater is the principle of using iPSC-derived mDA neurons for drug identification screens, something that has now been tested, although initially using iPSCs generated from a normal anonymous donor [40]. The greatest potential is for iPSC-derived cultures to facilitate an in vitro clinical trial, whereby the genetic diversity seen in the population can be captured in vitro, allowing the investigation of possible drug efficacy dependent on underlying genotype. It is already clear that PD is a heterogeneous disease: examples from a PD cohort in Cambridgeshire, U.K., include the influences of the MAPT (microtubule-associated protein tau) haplotype on secondary Parkinson’s disease: a clinician’s perspective. Exp. Neurobiol. 22, 77–83

References


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