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Perspectives of Parkinson's Disease Therapies using Induced Pluripotent Stem Cells

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ABSTRACT

Parkinson's Disease (PD) is an intractable disease resulting in localized neurodegeneration of dopaminergic neurons of the substantia nigra pars compacta. Many current therapies of PD are symptomatic, but no current option for clinical-grade disease modifying treatment exists. Fortunately, recent advances in the field of cellular reprogramming now allow for previously unattainable cell therapies and modeling of PD using induced pluripotent stem cells (iPSCs) to potentially restore a disease-free state. iPSCs can be selectively differentiated into a dopaminergic neuron fate to model endogenous physiology and pathogenesis. iPSC lines can also be established with genetically-linked PD. These patient-specific cell lines are then genetically corrected in mutations of influence and can be subsequently transplanted back into the patient to reestablish function. This year, induced pluripotent stem cells iPSCs entered the first human trial for PD therapy. This form of cell therapy has shown promising results in other model organisms and is currently one of our best options in slowing or even halting the progression of PD. Here we examine the genetic contributions that have reshaped our understanding of PD, as well as the advantages and applications of iPSCs for modeling disease and clinical therapies.

INTRODUCTION

Neurodegenerative diseases continue to pose increasing physical and financial burdens in an aging world, despite centuries of study. These disorders include Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Batten disease and Huntington's disease—among others. Since their discovery, neurodegenerative diseases have been clinically identified through post-mortem and physical examination. Formation of protein aggregates, localized neuronal death and progressive symptoms are all typical of neurodegeneration; however, our knowledge of the pathogenesis and etiology underlying these disorders has been rebuilt due to recent advances in the field of genetics. Spurred by regenerative medicine, research continues to search for underlying pathological mechanisms and therapies to halt or even slow the progression of these crippling diseases.

PATHOLOGY OF PARKINSON'S DISEASE

PD is the second most common neurodegenerative disease, debilitating 1% of the population over the last 60 years [1]. As such, it poses a special problem in aging societies [2,3]. Though many neuronal networks are affected by PD, it is the dopaminergic neurons (DAn) of the substantia nigra pars compacta (SNpc) [4-6] and nucleus Basalis of Meynert that are most acutely lost [7]. The localized cell death of the SNpc results in disruption of the basal ganglia's motor control

network, causing the characteristic motor symptoms of PD—bradykinesia, tremors, rigidity and other changes in speech and gait. PD's histopathology is marked by the presence of Lewy bodies and Lewy neurites that contain misfolded alpha-synuclein [8]. Lewy body formation typically begins in the SNpc, but a progressive spread to other structures in the brain has also been documented [9]. The role of alpha-synuclein has increased attention towards other non-motor symptoms of PD which include autonomic dysfunction, sleep disorders and neuropsychiatric symptoms (depression, psychosis, hallucinations) [10]. Many etiological questions remain. Although motor symptoms are only documented in 80% of revealed post-mortem alpha-synucleinopathy [11], these motor symptoms persist as a hallmark of clinical diagnosis.

PLURIPOTENT STEM CELLS

To better understand Parkinson's disease, researchers have sought models that mirror PD's phenotypic manifestations as closely as possible. To date, researchers have used model organisms (yeast, mice, drosophila and non-human primates) in three ways. First, organisms were subjected to intraperitoneal injections of 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) to mimic DAn cell death in the SNpc through oxidative stress. While helpful in examining the effects of blocking dopamine expression, this model fails to reconstruct the underlying neuropathology of highly-sensitive DAn and typical formation of Lewy neurites and Lewy bodies. Second, overexpression of the human risk factors, such as the SNCA gene, in model organisms has shown age-dependent DAn degeneration similar to the human pathological phenotype [8,12,13]. These results support a causal role for alpha-synuclein in PD progression, but currently lack clinical application. Finally, human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC) have been grafted into model organisms in an existing PD state. This procedure has shown mixed but promising results when executed with optimal methods of action [14-20]. Both hESCs and iPSCs possess unique qualities that make them ideal candidates for studying the development of PD [21]. Stem cells can be tailored to differentiate into a host of cell fates, including DAn of the SNpc that model PD on a cellular level [22,23]. Induced stem cells are also patient-specific, opening a window to the individual contribution of mutation and polymorphism risk factors on PD in a phenotypically similar state. DAn longevity can also be compared with other iPSC neurons such as cortical and olfactory neurons to probe the hypersensitivity of DAns specifically in the SNpc. Furthermore, the advent of TALEN, CRIPSR and other genetic reprogramming technologies have been applied to patient lines of iPSCs and extensively reviewed [24,25]. Corrected mutation lines can then be examined. Deriving a high percentage of fully functional, mature DAn of the ventral midbrain can be quite challenging and costly to scale up. The technical, in addition to ethical, obstacles of iPSC treatment may limit the feasibility of transplanting reprogrammed stem cells, but this opportunity in PD treatment is unprecedented. The first human clinical trial to transplant DAns from an iPSC source begins this year. Here we examine the discoveries leading to our current understanding of PD, as well as propose iPSC transplantation as one of the most viable forms of disease-modifying therapy for PD.

MAJOR GENETIC DISCOVERIES IN PARKINSON'S RESEARCH

By 2018, at least 5 major autosomal dominant genes, 5 autosomal recessive or X-linked factors and 11 monogenetic mutations for other disorders that present with Parkinsonian-like symptoms have been identified [26]. The most notable of these are mutations to and polymorphisms of LRRK2, which play a role in neuronal survival [27], and the SNCA gene, which affects alpha-synuclein production [28]. However, while strongly supported by a large body of statistical evidence [29], the effect of all known genetic mutations and risk-enhancing polymorphisms combined only explain a portion of the genetic risk of disease. Current advances in genetic probing will only allow for sharper analysis in genetic counseling, enhanced understanding of PD's progression and ultimately patient-specific treatments. In 1997, a novel, but rare, mutation was identified in the SNCA gene that coded for a relatively unknown protein called alpha-synuclein [28]. The missense mutation (A53T) resulted in autosomal dominant PD inheritance that could be tracked through the hereditary line with almost full penetrance. Found within Lewy bodies and Lewy neurites, alpha-synuclein expression has been attributed to PD's pathogenesis. The exact function of unaffected alpha-synuclein, thought to also assist in vesicle turnover and synaptic release, remains unknown [30]. However, one study suggests that alpha-synuclein, when properly folded into its tetramer, exhibits protective properties and slows Lewy body formation and aggregation [31]. In 2002, Funayma et al. reported that a region of chromosome 12 linked to PD inheritance in a Japanese family referenced as the Sagamihara kindred [32,33]. Two years later, the gene of interest was identified as LRRK2 [34]. Mutations to LRRK2 are by far the most common cause of genetic influence on PD [33,35]. LRRK2 mutations comprise 4% of reported familial PD, and most cases exhibit pathology indistinguishable from sporadic PD with both Lewy body formation and DAn death [34-36]. Studies in cellular models that harbor these mutations show increased kinase activity resulting in neuro-oxidative stress and toxicity [37,38]. Although the protein is multifunctional, LRRK2 knock-downs inhibit differentiation from neural progenitors to DAns and increase cell death [27]. These findings suggest LRRK2's facilitation in cell survival and differentiation in the ventral midbrain.Genetic loci have also been identified in familial PD that follow autosomal-recessive inheritance. Two genes, phosphate and tensin homolog-induced putative kinase 1 (PINK1) and Daisuke-Junko-1 (DJ-1), are of special interest. In cases of homozygosity, both PINK1 and DJ-1 mutations result in very early onset in the 30's, low response to levodopa treatment and slow disease progression [39,40]. Additionally, an astute clinical

observation of the comorbidity between Gaucher disease (GD) and PD led researchers to examine other proteins with suspect. GD is an autosomal-recessive disease resulting from homozygous mutations to GBA. GBA, a lysosomal enzyme of the CNS, is thought to also have a role in protein aggregation in PD when mutated [41]. GBA mutations have been proven in 2009 to be the most common genetic risk factor for PD so far—present in 3-7% of idiopathic PD cases [42]. Technological advances combined with ever-increasing sample sizes and collaboration will hopefully uncover more sources of genetic and epigenetic influence. A summary of the genes discussed above are summarized in **Table 1**.

Table 1: Major Familial Forms for and Genetic Factors to PD.

PD Inheritance	Disease (Mutations)	Gene Location	Gene Function	Phenotype
Autosomal Dominant	PARK-SNCA, (A53T,A30P,E46K,H50Q,G51D,duplication,triplication	SNCA, 4q22-1	The SNCA gene encodes for the alpha sysnuclien protein that is widely expressed in presynaptic terminal of neurons.Alpha-sysnuclein maintains the production of vesicles involved in neuronal communication.Alpha-sysnuclein is also thought to play a role in dopamine expression of voluntary and involuntary movement pathways	Early-onset PD. Neurodegeneration within the SNpc and Lewy Body information throughout the brain.
	PARK-LRRK2,(G0219s,R1441C)	LRRK2,I2q12	Encodes the Leucin rich repeat kinaase 2 protein, expressed in the cytoplasm and mitochindrial membrane of neurons. LRRK2 is heavily involved in the ubiquitination of molecules, leading to their degradation. The price function in PD is not known, but it is thought to coordinate neuronal survival and differentiation in the midbrain.	Late-onset PD with mixed neuropathology. Some cases present with Lewy body formation and DAn death in the SN, others without Lewy Body formation
Autosomal Recessive	PARK-DJ1, (Q456X, V170G)	DJ-1. 1p36.23	Encodes the protein DJ-1, found in the brain and other tissues throughout the body. DJ-1 is a multifunctional protein with roles involved in the prevention of alpha-sysnulein aggregation, neuronal protection under conditions of the oxidative stress, transcriptional regulation and prevention of metal-induced cytotoxicity. All or some of this functions may be involved in sometypes of early PD formation	Conclusive data has not been reported.
	PARK-PINK1, (exon 7 deletion)	PINK1,Ip36.12	Codes for the protein PTEN-induced putative kinase 1, located within mitochondria. PINK1 exhibits a protective function of mitochondria	Early-onset PD complete with Lewy body formation and acute DAn loss in the SNpc.

			during cellular stress by causing the parkin protein to bind the deploarized mitochondria and induced autophagy.	
Genetic Risk Factor	Gaucher disease (L444P, N370S)	GBA, 1q22	Codes for an enzyme active in lysosomes and cellular membranes. Beta-glucocerebosidase is a housekeeping enzyme hydrolyzes the beta glucosidic linkage of glucocerebroside into glucose abd ceramide. Mutation causes glucocerebroside buildup in macrophages.	Several neurological complications in addition to liver failure, bone lesions and low blood cell counts.

iPSCs AS DISEASE MODEL FOR PARKINSON'S DISEASE

The contribution of genetics has been heightened through the use of patient-specific iPSC lines and genetic engineering technology to manipulate them. Ever since Yamanaka's discovery in 2007 that a handful of transcription factors can rewind cellular differentiation, iPSCs have been utilized extensively in the study of neurodegenerative disease to direct patient-specific cell fate [43,44]. While still limited in scope, iPSCs are currently the most robust and phenotypically similar model for PD [45]. Mutations of consequence can now be captured in iPSC lines and directed by small molecules to a DAn fate in PD models—all within a dish. Displayed openly, the real-time cellular effects of mutation can be physically observed and studied in tandem with control lines to limit genetic background effects of the affected individual; similarly, effects of oxidative stress common to PD can also be quantified with broad clinical applications for drug screening without human side-effects. Not surprisingly, it remains difficult to physically confirm the mechanisms of neurodegeneration and neuroprotection implicated by iPSC research as patients' neurons are hidden deep within the brain. These effects similarly cannot be perfectly translated into the cellular environment of PD due to some epigenetic effects of aging eliminated in reprogramming protocols. Differentiation of iPSCs to midbrain DAns begins with the initial dedifferentiation. In the last 10 years, thousands of iPSC lines have been generated by overexpressing certain transcriptional factors in somatic cells to bring them back to a pluripotent state. Those methods have been extensively reviewed [24,25]. Current methods of reprogramming employ episomes, viruses and synthetic mRNA to upregulate expression of the transcription factors without genomic integration that leads to tumorigenesis [46-48]. Dedifferentiation often results in mutation and, consequently, not all iPSC lines are of equal quality. Though iPSC reprogramming theoretically results in a clonal copy of the genome, sequencing entire genomes of iPSCs have revealed an average of 6 de novo mutations during reprogramming in coding regions [49]. Extensive quality controls are in place to measure the quality of iPSC lines such as NANOG expression, transcriptome analysis of iPSC lines with available data of hESC expression as well as bioinformatics tests like Pluritest [50]. These safeguards are critical if clinicians wish to implement iPSCs further in personalized medicine. Differentiation from pluripotent stem cells to mature DAns of the midbrain mimics a specific pathway in embryological development. Initially dopaminergic neurons of the SNpc were originally thought to have derived from neuroepithelial cells like other cortical neurons, but in fact they are similar to the spinal cord, derived from the ventral floor plate of the neural tube [51]. The embryologic origin was confirmed by expression of other floor-plate markers such as Lmx1a and FoxA2 [52,53]. Differentiation protocols using small molecules and neurotrophic factors mimic in vivo neural floor-plate patterning by activating the sonic hedgehog (SHH) pathway, inhibition of SMAD and addition of FGF8 [54,55]. Differentiation through transfecting transcriptional factors can also be achieved, but spontaneous integration prohibits these methods from any clinical-grade application. Tuning DAn cell type is achieved with the addition of the WNT signaling molecule CHIR99021 (CHIR). The more CHIR added, the more hindbrain characteristics DA neurons adopt [56]. With the correct amount, DAns of the SNpc can be achieved that express characteristic GIRK2 markers. Neurogenesis of localized DAns in iPSC lines provides unparalleled modeling of human conditions in PD. In stem cell models, unlike other model organisms, endogenous cellular machinery and transcriptional feedback are preserved, a fundamental step in accurately modeling this genetically complex disease. iPSCs have also been used to model AD, suggesting broader applications to a whole range of neurodegenerative disorders [57]. Furthermore, iPSC lines can now be maintained with a natural susceptibility to PD pathology without unnaturally high oxidation from MPTP. Genetic effects may be further isolated by implementation of CRISPR/Cas9 editing to reduce genetic and clonal variation. iPSC mutation models can be additionally genetically corrected at dedifferentiation and co-cultured with mutant lines to control for epigenetic and passage state [58]. Though reprogramming technologies have been used on patients with idiopathic PD, an iPSC model offers the greatest genetic insight into patients with

monogenetic causes. In 2011, the first iPSC line with genetically linked PD was established with a A53T mutation in the SNCA gene [59]. The mutation was subsequently fixed and co-cultures were both differentiated to tyrosine hydroxylase positive (TH+) neurons. Dozens of lines have also been taken from members of the Iowa kindred with duplications and triplications of the SNCA gene [60]. These lines have shown increased sensitivity to neurotoxins and oxidative stressors, indicating a more accurate model of PD, but with healthy skepticism as the addition of toxins may not accurately portray the underlying mechanisms of PD [21]. Nevertheless, these models are useful in exploring affected patients' endogenous response to environmental damage, possibly indicating mitochondrial malfunction. While this list is in no way exhaustive, genetic susceptibility has also been quantified in multiple iPSC lines with LRRK2 [61-63], PINK1 [64] and GBA mutations [65]. Perhaps iPSC technology will also be used as a diagnostic tool in the future to predict individual susceptibility to PD as well as a sourcing cell therapy. The predisposition of DAn death in the ventral midbrain had long eluded models, but a new generation of iPSC mutant lines meets the challenge. Additionally, iPSC lines are established without the sacrifice of human zygotes or damaging side-effects in drug trials, allowing for the investigation of pathology without human harm or ethical concerns. These conditions are all foundational to two treatments only now within reach: cell therapy and patient-specific transplantation.

iPSCs AS CELL THERAPY FOR PARKINSON'S DISEASE

Initial treatment of PD started in 1960 with the discovery that affected individuals lacked neurological dopamine. Clinicians began administering intravenous levodopa, a precursor to dopamine that can pass the blood-brain barrier, with almost immediate improvement of symptoms [66]. Levodopa treatment remained the gold standard of treating PD for decades, but currently there are other commercially available medications that target PD without a dopaminergic mechanism of action [67]. Increased focus on the dopaminergic pathway also helped in illuminating the motor circuitry of the basal ganglia. With new understanding of the affected circuits, deep brain stimulation (DBS) by electrical stimulation to the internal segment of the globus pallidus and subthalamic nucleus was introduced as a supplemental therapy with dramatically positive results [68]. Time in the field of DBS has only improved precision of electrode placement, higher flexibility and longer battery life to curb side-effects [68-70]. Like most neurological disorders, a number of studies have also shown the relative effectiveness of non-medical, non-surgical interventions such as exercise, dance and meditation [71,72]. Sparked by the genetic revolution, revealed subcellular mechanisms finally allow for a shift in focus from symptomatic therapies to the development of clinical-grade disease modifying treatment. Not for lack of interest, no disease-altering therapeutic options are currently available that can slow or halt the neurodegeneration of PD. However, there are a handful of drug candidates that show promise in varying stages of clinical trials [73]. The effectiveness of iPSCs has also been examined as a method to achieve such disease-altering treatment. Stem cells have already been used as therapy in a number of trials involving neural damage. The first occurred in 2010 with a clinical trial that used hESCs to treat spinal cord injury (SCI) [74]. Since then, stem cell-derived products have been used in other animal and human trials for therapy of neural disorders ranging from positive results with age-related macular degeneration (AMD) to poor results in AD [75,76]. These trials produced mixed results— in some cases highlighting the limitations of model organisms, due to cellular and gross anatomical differences, to predict efficacy of treatment in human neurodegeneration [77]. With relatively localized neurodegeneration, PD is also a good candidate for cell therapy. Fetal tissue of the ventral midbrain was implanted in PD patients in 1987, and results from the trial showed cell survival and DAn functionality even 20 years after implantation in some cases [78,79]. As discussed above, the ethics of harvesting 4-10 embryos per patient and limitations to cell survival after preservation prevents fetal cell grafts as a viable form of cellular therapy on a national scale [80]. hESCs are also being utilized in a 2017 Chinese trial for PD, but it is too early to speculate on its effectiveness without conclusive data [81]. Though hESC implantation may produce promising results, strong immunosuppressants must be used to ensure that the graft is accepted. Safety in transplantation of all reprogrammed cells is paramount. Precautions must be taken to prevent infection, graft-induced dyskinesia and tumorigenesis when transplantation trials are conducted. Though cellular transplantation always poses some risk, the advantages of iPSCs present a viable future for cell therapy of PD. The history of these pertinent cell-based therapies can be visualized in **Figure 1**.

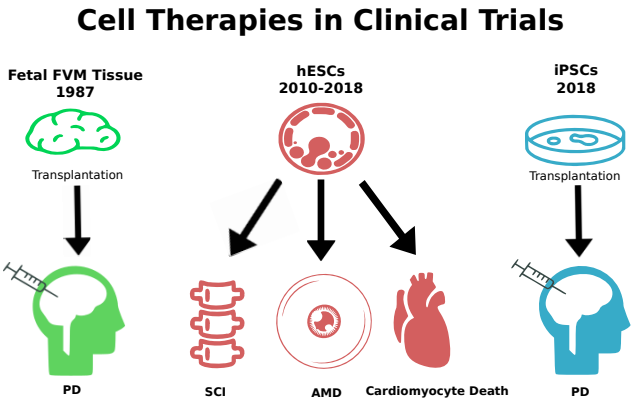

Figure 1: The progression of pluripotent cell-based therapies within the context of PD research. Beginning in 1987, fetal ventral midbrain DAns were used as the cell source for the first clinical trial using cells to treat PD. In recent years, hESCs are being utilized in a number of clinical trials involving neurodegeneration. Use of hESCs has shown special promise in spinal chord injury (SCI), age-related macular degeneration (AMD) as well as cell damage to the heart. In the summer of 2018, clinicians are beginning to undertake the first human trial using iPSCs as a cell source to treat PD. This trial will follow seven patients over the course of two years. The outcomes of these trials are detailed in the text.

The first advantage is that iPSC lines can be established without the sacrifice of human embryos, removing a large ethical obstacle of human stem cell treatments. iPSCs also permit human leukocyte antigen (HLA) matches in patient-specific treatments, effectively reducing the severity of post-operational immunosuppressants. Histocompatibility has additionally shown reduced immune response of lymphocytes and microglia as well as increased cell survival in iPSC transplantation of DAns in primate studies [82]. iPS dedifferentiation and reprogramming may be lengthy and burden the patient with high cost, but reduced immune rejection and generic donor lines could significantly reduce costs when scaled up. The steps required for patient-specific transplantation of iPSCs are outlined in **Figure 2**.

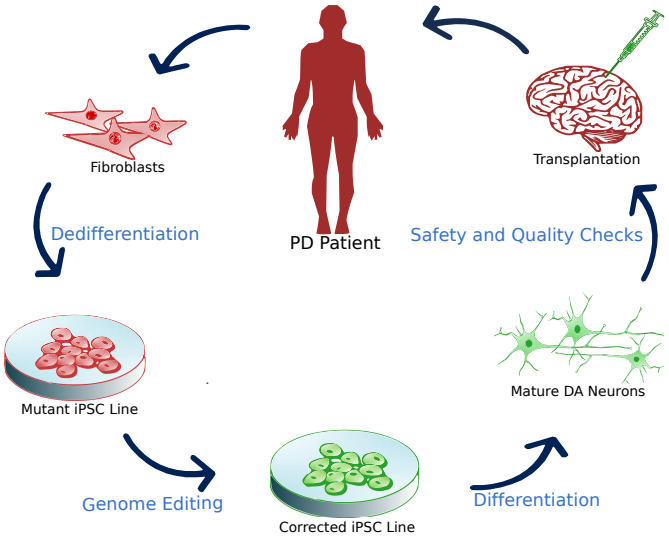

Figure 2: iPSC transplantation. First, fibroblasts are obtained from a patient afflicted with familial PD. Researchers express major reprogramming transcription factors to establish a mutant iPSC line. Using ZNF/TALEN or CRISPR/Cas9 technology, the significant mutation is corrected and then the line is differentiated into mature or progenitor DA neurons in xeno-free conditions. After sufficient quality assurance, the differentiated cells can then be used for clinical trials.

In Japan, researchers estimate that 50 iPS lines from HLA-homozygous donors will cover 73% of the Japanese population by matching three HLA loci (A, B and DR) [47]. Primate studies have already demonstrated significant improvements after two years of treatment of PD using iPSCs [83]. But clinical efficacy of treating PD with iPSC grafts in humans will be established in the approaching future. A team headed by Takahashi announced the first human clinical trial of iPSC-generated DAn transplantation to treat PD [84]. The trial began August 1st, 2018 at Kyoto University Hospital. Cells will be sourced from third-party donors with matching HLA loci to ensure genetic integrity and eliminate the patients' genetic interference. However, patients will still receive immunosuppressants due to the trial's exploratory nature. Approximately 5 million cells will be administered to the SNpc through two drilled holes in the skull [85]. Seven patients with moderate PD have been selected for the trial with the benefit of cell therapy earlier on in neurodegeneration but also posing greater experimental risks and will be followed for two years. Clinicians will monitor the progression of the

disease, as well as other side effects of too much dopamine in the SNpc that would result in involuntary movements. While many questions and ethical issues surrounding iPSCs and genetic engineering remain, the future for PD looks promising. However, iPSC treatment of PD will likely not completely restore function and should be examined within the context of other treatment options.

CONCLUSION

The loss of even a small cluster of 7,800 DAns in the SNpc may result in severe debilitation in PD patients. Though cell death may arise from a number of postulated mechanisms, ultimately neuron survival is integral for proper motor and cognitive function. With no current therapies to recover from critical cell death, iPSCs provide an alternate route to potentially restore a disease-free state. New, patient-specific cells that are not predisposed to PD may be transplanted back into the SNpc, an ambition to restore function finally within reach. Programmed cell death does not afflict PD patients alone; iPSC therapy may alleviate this cell death associated with broader applications for AD, ALS and Huntington's disease.

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