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Modeling Parkinson's disease with induced pluripotent stem cells harboring α-synuclein mutations

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Abstract

Parkinson's disease (PD) is a common neurodegenerative condition affecting more than 8 million people worldwide. Although, the majority of PD cases are sporadic in nature, there are a growing number of monogenic mutations identified to cause PD in a highly penetrant manner. Many of these familial mutations give rise to a condition that is clinically and neuropathologically similar, if not identical, to sporadic PD. Mutations in genes such as *SNCA* cause PD in an autosomal dominant manner and patients have motor and non-motor symptoms that are typical for sporadic PD. With the advent of reprogramming technology it is now possible to capture these mutations in induced pluripotent stem cells (iPSCs) to establish models of PD in a dish. There are multiple neuronal subtypes affected in PD including the midbrain dopaminergic (mDA) neurons of the *substantia nigra*. Robust neuronal differentiation into mDA or other relevant neural cell types are critical to accurately model the disease and ensure the findings are relevant to understanding the disease process. Another challenge for establishing accurate models of PD is being met by the generation of isogenic control iPSC lines with precise correction of mutations using advanced gene editing technology. The contributions of ageing and environmental factors present further challenges to this field, but significant progress is being made in these areas to establish highly relevant and robust models of PD. These human neuronal models, used in conjunction with other model systems, will vastly improve our understanding of the early stages of the PD, which will be key to identifying disease-modifying and preventative treatments.

Familial Parkinson's disease

William Gowers was the first to report that Parkinson's may be hereditary. He noted that although less than 15% of his patients had a family history of PD, but when it did exist, multiple family members were affected prompting him to state, "hereditary influence may be powerful" (1). In 1997 the first genetic cause of PD was identified to be a G209A mutation in the *SNCA* gene, encoding a relatively unknown protein at the time α-synuclein (2). This resulted in a substitution of alanine to threonine at position 53 of the protein (A53T). The mode of inheritance was autosomal dominant and the mutation appeared to be a fully penetrant cause of disease in the affected families (2). The significance of this important genetic discovery was further enhanced with a report demonstrating that aggregated α-synuclein was a major component of Lewy bodies, the pathological hallmark of sporadic PD (3). Since then five additional mutations in the *SNCA* gene have been identified to cause familial PD. Each mutation caused a single amino acid substition to α-synuclein: A30P, E46K, H50Q, G51D, and A53E (4-10). In addition to point mutations, duplication or triplication of a region within chromosome 4q21 encompassing the *SNCA* gene locus, were identified to cause autosomal dominant PD (11-13). The size of the

duplication or triplication region differs between families suggestive of *de novo* chromosomal events (14). The dose of α-synuclein over-expression is positively correlated with clinical severity, with *SNCA* duplication patients exhibiting a later age of onset and a slower rate of progression than *SNCA* triplication patients (14,15).

The most common cause of familial PD is due to mutations in the gene encoding LRRK2 (leucine-rich repeat kinase 2) (16,17). This is a large multifunctional protein and familial mutations are thought to increase its kinase activity (18). The *LRRK2* mutations are autosomal dominant with age-dependent penetrance. Patients with *LRRK2* mutations have Levodopa-responsive PD and Lewy body pathology that is similar to sporadic Parkinson's. The heterogeneity of LRRK2 genetics and its implications for therapeutic approaches to PD have been reviewed (19). In the last 20 years, at least 12 additional genes have been identified to cause familial PD when mutated (20). Some of the mutations cause Lewy body-positive PD with a disease course and progression similar to sporadic PD, while other mutations, including the majority of autosomal recessive mutations, cause an atypical PD that is often negative for Lewy body formation (21).

Reprogramming and neuronal differentiation

The discovery that adult cells can be reversed in developmental time to an embryonic state by the over-expression of a handful of transcription factors has been transformative (22,23). This so-called reprogramming technology has provided a unique and unprecedented opportunity to model human conditions with disease-relevant cell types. Although patients with idiopathic disease have been studied with reprogramming, this technology is most powerful when applied to patients with a monogenic or complex genetic cause to their condition. The methods to reprogram somatic cells, such as blood or skin, into induced pluripotent stem cells (iPSCs) have been extensively reviewed (24,25). The current and most common methods for human iPSC generation use non-integrating reprogramming technologies, such as episomes, synthetic mRNAs or Sendai virus (26-28). There have been thousands of human iPSC lines generated worldwide over the last 10 years, and it is now clear that not all iPSC lines are created equal. The issues of partial reprogramming or epigenetic memory of the starting somatic cell type may hamper any downstream disease modeling efforts. There are numerous quality-control measures that can be applied to measure the quality of the iPSCs produced. Morphology and expression of pluripotent markers, such as NANOG, are useful as a first level of screening, but these measures cannot distinguish good from poor iPSC. However, one of the most robust measures of iPSC quality is transcriptomic analysis. The global transcriptional signature of a high-quality iPSC line should be within the transcriptional 'space' of bona fide human embryonic stem cells (hESCs). This can be accomplished with bioinformatics tools such as PluriTest (29), or by principle component analysis comparing iPSC transcriptome data with publicly-available data on hESCs (30). If the RNA expression signature of an iPSC line does not cluster with hESCs, it suggests either incomplete reprogramming or the cell line has novel properties that are outside the normal phenotype of a human pluripotent stem cell line. One of these properties could be a reduced ability to differentiate into the three germ layers, which will hamper any downstream disease modeling. Variable differentiation potency of iPSC lines, even from the same individual, have been observed in studies that use sufficiently large amounts of iPSC lines (31,32). The teratoma assay is commonly used to characterize novel iPSC lines, but low-quality iPSCs with an aberrant transcriptional signature and poor differentiation capabilities perform well in this assay. The ability to form teratomas is not a reliable method to discriminate between high and low quality iPSCs.

The other important aspect of reprogramming to be concerned with is the genetic integrity of the established iPSC lines. Since human iPSC generation is usually a clonal process from an adult cell type, there are many opportunities mutations or chromosomal aberrations to occur *de novo*, or be expanded from the somatic cell starting material. Whole genome sequencing of a large collection of iPSC lines demonstrated that an average of 6 coding mutations occur during reprogramming (33), and whole genome SNP arrays showed that novel copy number variations arise in iPSCs with high frequency (34). Large chromosomal abnormalities are also fairly common during reprogramming and subsequent expansion of cells with over 20% of published iPSC lines harboring a karyotypic defect (35). These findings highlight the need for multiple iPSC lines and mutation-corrected isogenic control lines for each genetic condition under investigation.

Once a collection of disease and control iPSC lines have been established and validated, the next stage is to differentiate them into specific cell types relevant to the condition being modeled. For PD this is most often midbrain dopaminergic (mDA) neurons. However, it is equally valid to differentiate iPSCs into other neuronal cell types affected in PD, such as enteric neurons, olfactory neurons, and cortical neurons. The rate-limiting step is the availability and robustness of protocols to produce these various cell types. Cortical neurons can be efficiently differentiated from human iPSCs in the presence of inhibitors of the two major branches of the SMAD signaling pathway – (i) BMP and (ii) TFGβ/activin (36). This so-called dual SMAD inhibition protocol results in the production of multiple classes of cortical neurons representative of all the cortical layers (37). These neurons would be suitable for investigating the dementia aspects of PD and for modeling dementia with Lewy bodies.

Although many neuronal systems are affected in PD, it is the mDA neurons of the *substantia nigra pars compacta* (SNpc) that are most acutely lost. Protocols to produce mDA neurons from pluripotent stem cells were relatively inefficient until the focus shifted to floor plate tissue. The embryological origin of nigral dopaminergic neurons is the floor plate of the midbrain. While this signaling centre in the ventral neural tube would normally degenerate in the spinal cord and hindbrain, it survives in the midbrain and gives rise to mDA neurons (38). Based on this knowledge from embryo development, human floor plate cells were efficiently differentiated from hESCs using dual SMAD inhibition and early, strong activation of the sonic hedgehog (SHH) pathway (39). These cells were similar to diencephalic floor plate, but could be caudalized by factors, such as WNTs and retinoic acid. This protocol was refined with the use of the GSK3β inhibitor, CHIR99021 (CHIR), to activate the WNT signaling pathway to produce midbrain floor plate (40). This floor plate tissue could be further differentiated into mDA neurons and matured *in vitro* to express markers of the SNpc, such as GIRK2, and exhibit electrophysiological properties similar to SNpc neurons *in vivo*. Furthermore, when mDA progenitors from this floor plate protocol were grafted into 6-hydroxy-dopamine (6-OHDA) lesioned mice and rats, the cells survived and matured into functional mDA neurons, and rescued the dopamine deficits in these animal models (40). Tuning the level of CHIR is critical to producing the desired mDA neuronal cell type. Too much CHIR and the cells become hindbrain neurons, and too little CHIR the neurons take on more rostral fates (41). Most of these protocols have been optimized with one or two hESC lines, and transfer to other cell lines often requires adjustments to the protocol, or in extreme cases the protocol fails to work with some cell lines (31,42). It is important for disease modeling to be aware of variable differentiation propensity of iPSC lines, and to control for this by identifying, and quantifying, the neuronal and non-neuronal cell types in each batch of differentiated cells (Figure 1A). Optimization of differentiation conditions for each iPSC line under investigation may be necessary.

iPSCs with α-synuclein mutations to model Parkinson's

The first report of familial PD iPSCs with an α-synuclein mutation was from Rudolf Jaenisch's laboratory. They established iPSC lines from a skin biopsy from a patient in the Contursi kindred harboring the A53T *SNCA* mutation (43). Furthermore, they used zinc finger nucleases to repair the point mutation and establish isogenic control lines. They also introduced this mutation, and separately the E46K *SNCA* mutation into wild-type hESCs to establish additional sets of disease and control isogenic cell lines (43). They demonstrated that the cell lines could differentiate into tyrosine hydroxylase (TH)-positive dopaminergic neurons, but phenotypic analysis was not undertaken (Table 1).

The second reported set of iPSCs with an α-synuclein mutation was from a female patient of the Iowa kindred harboring a triplication of the *SNCA* locus. Devine and colleagues established 30 iPSC lines from the alpha-synuclein triplication (AST) patient, and 10 iPSC lines from her unaffected daughter (NAS – for normal alpha-synuclein) (42). Due to the large number of cell lines produced, this was one of the first publications to observe variability in differentiation propensity between iPSC lines derived from the same individual. When differentiation efficiency was controlled for, a doubling of *SNCA* mRNA and α-synuclein protein was observed in AST mDA neurons compared to control neurons, as predicted from the genotypes. While verifying that the triplication mutation was present, it was confirmed that the multiplication region on chromosome 4q22 was very large indeed (>1.9 Mb), and encompassed at least 9 coding genes including *SNCA* (14). Gene expression profiling revealed that all genes in the triplication region, including α-synuclein, were over-expressed in AST mDA neurons when compared to control neurons. There is another family in the US, the Lister kindred, which harbors a much smaller triplication region that encompasses *SNCA*, and one other gene, *MMRN1*, but they have the same clinical and pathological features as the Iowa kindred (14). These observations, as well as data from duplication *SNCA* families, strongly implicate increased α-synuclein expression as the cause of familial PD in these families. However, the over-expression of multiple proteins in the disease *versus* control neurons will complicate the interpretation of results when performing phenotypic analysis in iPSC-derived neurons *in vitro*. Isogenic control lines could be generated by precisely deleting two of the four *SNCA* alleles, while leaving the rest of the triplication region intact (Figure 1B). This is expected to reduce the expression of α-synuclein to wild-type levels, while leaving the other linked genes in the triplication over-expressed. Phenotypes specifically due to elevated α-synuclein expression should be rescued in such isogenic control lines.

Another set of triplication *SNCA* iPSC lines were established from a male member of the Iowa kindred and control iPSCs from his unaffected sister (44). Multiple iPSC lines were differentiated into mDA neurons using a rosette-based protocol (45). They also observed elevated expression of *SNCA* mRNA and α-synuclein protein in triplication *SNCA* neurons, as well as increased expression of a select set of oxidative stress genes and aggregation-related genes, HMOX2, DNAJA1, and HSPB1 (Table 1). Furthermore, triplication SNCA neurons had increased sensitivity to hydrogen peroxide treatment (46).

Susan Lindquist and colleagues used a yeast model of synucleinopathy to identify vesicular trafficking defects and nitrosative stress as major phenotypic outcomes of aggregated α-synuclein protein (47). These insights were used to guide phenotypic studies in A53T *SNCA* and triplication *SNCA* iPSC models. They observed significant levels of nitrosative stress in A53T *SNCA* cortical neurons, which was absent in isogenic, mutation-corrected control neurons (48). Endoplasmic reticulum (ER) stress was also observed in A53T and triplication *SNCA* neurons with the accumulation of ER-associated degradation (ERAD) substrates (Table 1). This phenotype was not observed in isogenic control neurons or in hESC-derived cortical neurons. Furthermore, drugs identified in a yeast screen, such as NAB2, to alleviate phenotypes due to α-synuclein aggregation, also reduce levels of ER and nitrosative stress in human A53T *SNCA* neurons underscoring the conserved mechanisms of toxicity for this protein (48).

Flierl and colleagues investigated two triplication *SNCA* iPSC lines from a male member of the Iowa kindred, and a control iPSC cell line from his unaffected sister (49). They also established an *SNCA* knock-down line with some reduction of α-synuclein in the triplication *SNCA* line. All of the phenotypic analysis was performed in neural precursor cells (NPCs) established by the dual SMAD inhibition method. Phenotypes included reduced S-phase, increased sensitivity to the toxins rotenone and paraquat, and increased reactive oxygen species production (Table 1). It is unclear if one or both of the triplication SNCA iPSC lines exhibited these phenotypes, but it is compelling to see that knock-down of *SNCA* expression in NPCs by RNAi rescued a number of the defects (49). A subsequent publication using the same set of iPSC lines observed a defect in neuronal differentiation and maturation of the triplication *SNCA* NPCs (50). Knock-down of *SNCA* expression partially rescued the differentiation defect in one of the two iPSC lines. The small number of iPSC lines used in this study, and the lack of a differentiation defect in other studies on triplication *SNCA* iPSC lines suggest the observed developmental phenotype is likely due to clonal variation in differentiation propensity.

Lin and colleagues examined a total of 12 iPSC lines, including a triplication *SNCA* line, several *LRRK2* mutant lines, and a number of control lines, and found no significant difference in efficiency of mDA differentiation (51). However, they did observe significant accumulation of phospho-Ser-129-α-synuclein (pSer129-αSyn), a pathological mark, in triplication *SNCA* neurons, but not in *LRRK2* mutant neurons (51). Similar to other studies, they also observed an increased sensitivity of the PD neurons to toxins, such as 6-OHDA and paraquat. RNAseq revealed altered expression in genes involved in synaptic signaling transmission, as well as genes involved in alternative splicing, the latter likely due to the up-regulation of RBFOX1 (51).

Most recently Rebecca Matsas and colleagues reported defects in synaptic connectivity and axonal pathology in neurons differentiated from multiple A53T *SNCA* iPSC lines established from two patients (52). Similar to the triplication *SNCA* neurons, they detected pSer129-αSyn structures in A53T *SNCA* neurons, but not in control neurons. They also identified Thioflavin S-positive aggregates, α-synuclein-positive structures within inclusion bodies and reduction of neurite quantity and length. RNA expression profiling identified a preponderance of differentially expressed transcripts encoding for pre-synaptic and post-synaptic proteins (52). In the absence of isogenic control iPSC lines, they used small molecule modulators of α-synuclein aggregation, such as NPT100-18A. This is a cyclic peptidomimetic compound that was shown to reduce α-synuclein accumulation and phosphorylation in transgenic mice and it can rescue motor deficits in multiple PD rodent models (53). Treatment of A53T *SNCA* iPSC-derived neurons with NPT100-18A significantly rescued the neurite defects and reduced the sensitivity of the neurons to oxidative stress (52).

Summary

Numerous studies on iPSCs harboring the A53T SNCA mutation or triplication of the wild-type SNCA gene are now published (42-44,48-52). The over-expression of SNCA mRNA and α-synuclein protein is a common observation across multiple studies on triplication SNCA iPSCs, as well as increased sensitivity of oxidative stress, which may be indicative of mitochondrial dysfunction. The early pathological PD marker, pSer129-αSyn, was investigated and identified in both triplication and A53T *SNCA* neurons (51,52). ER stress and alterations at the synapse were also observed and in agreement with other cell and rodent models of α-synuclein dysfunction. Gene expression profiling also pointed to the synapse as an early target of α-synuclein dysfunction.

Modeling synucleinopathies with iPSC-derived neurons is challenging and requires an appreciation of the clonal variation of iPSC lines and of the heterogeneous and variable

outcome of differentiation protocols. With the advent of CRISPR/Cas9 genome editing it is now feasible to establish isogenic control lines to reduce problems of genetic background as well as clonal variation. Furthermore, it is now possible to simultaneously generate mutation-corrected isogenic control iPSCs lines during the initial reprogramming process (54). This not only saves time, but also ensures that the mutant and isogenic controls lines are at a similar passage and epigenetic state. Mutations that cause late-onset neurodegenerative conditions, such as PD, are not expected to cause developmental defects. Therefore, differentiated populations should be quantitatively characterized for the presence of desired cell types to ensure the iPSC-derived neurons are of the same identity and at similar levels of maturity prior to phenotypic analysis.

Table 1. Publications on human iPSCs with *SNCA* mutations

| Study	Mutant iPSCs	Control iPSCs	Differentiation	Phenotypes
Soldner et al 2011	A53T SNCA iPSCs	Isogenic-corrected A53T SNCA iPSCs	Midbrain dopaminergic neurons	N/A
Devine et al 2011	triplication SNCA iPSCs	1st-degree, unaffected relative iPSCs	Midbrain dopaminergic neurons	Elevated levels of SNCA mRNA, cellular and secreted aSyn protein
Byers et al 2011	triplication SNCA iPSCs	1st-degree, unaffected relative iPSCs	Midbrain dopaminergic neurons	Elevated aSyn protein expression, increased expression of oxidative stress genes, and increased susceptibility to oxidative stress
Chung et al 2013	A53T SNCA, triplication SNCA iPSCs	Isogenic-corrected A53T SNCA iPSCs, hESCs	Forebrain cortical neurons	Nitrosative stress, accumulation of ER-associated degradation (ERAD) substrates, ER stress
Flierl et al 2014	triplication SNCA iPSCs	triplication SNCA-knock-down iPSCs	Neural precursor cells	High vulnerability to stress, increased ROS production
Oliveira et al 2015	triplication SNCA iPSCs	triplication SNCA-knock-down iPSCs	Neural precursor cells, dopaminergic neurons	Impaired neuronal differentiation and maturation
Lin et al 2016	triplication SNCA iPSCs	Unaffected, unrelated iPSCs	Midbrain dopaminergic neurons	pSer129-aSyn accumulation, increased suspectibility to oxidative stress
Kouroupi et al 2017	A53T SNCA iPSCs	Unaffected, unrelated iPSCs	Dopaminergic, GABAergic, and glutamatergic neurons	protein aggregation (Thioflavin S, pSer129-aSyn), axonal neuropathology, and altered expression of synaptic transcripts
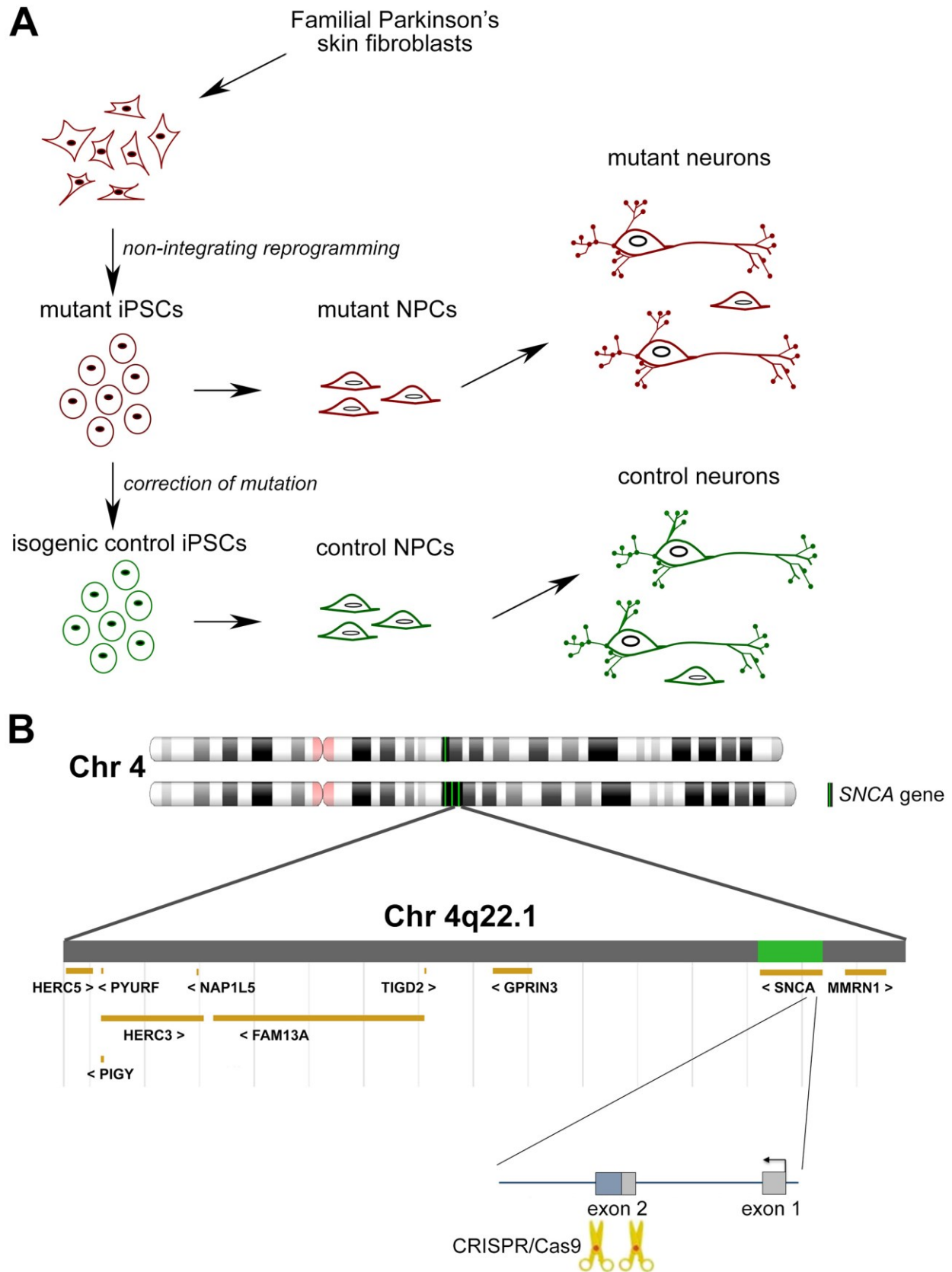
Figure 1. Modeling Parkinson's with mutant and isogenic control iPSC lines.
(A) Skin fibroblasts (or blood) from a familial Parkinson's patient is used to generate iPSC lines using a non-integrating method of reprogramming. Monogenic mutations can then be corrected by CRISPR/Cas9 technology to produce isogenic control iPSC lines. The mutant

and control iPSC lines are then differentiated into neural precursor cells (NPCs), and matured into neurons using optimized protocols. It is important to confirm differentiation efficiency is relatively similar for the different iPSC lines. (B) The triplication *SNCA* mutation that causes autosomal dominant PD is located on the long arm of chromosome 4. The triplication region is greater than 1.5 Mb in size and encompasses multiple coding genes in addition to *SNCA*. These genes are *MMRN1, GPRIN3, TIGD2, FAM13A, NAP1L5, PIGY, PYURF, HERC3,* and *HERC5*. In order to generate isogenic control iPSC lines, the first coding exon of the *SNCA* gene (exon 2) can be deleted by CRISPR/Cas9 technology without disrupting the rest of the triplication region.

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