HHS Public Access

Author manuscript

Stem Cell Res. Author manuscript; available in PMC 2024 June 01.

Published in final edited form as:

Stem Cell Res. 2023 June ; 69: 103125. doi:10.1016/j.scr.2023.103125.

Generation of gene-corrected isogenic controls from Parkinson's disease patient iPSC lines carrying the pathogenic *SNCA* p.A53T variant

Nikita Kozhushko,

Aleksandra Beilina,

Mark R Cookson

Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda, MD 20892

Abstract

Pathogenic variants in the alpha-synuclein (*SNCA*) gene cause familial forms of Parkinson's disease (PD). Here, we describe generation of six isogenic controls from iPS cell lines derived from two PD disease patients carrying the *SNCA*p.A53T variant. The controls were created using CRISPR/Cas9 technology and are available for use by the PD research community to study A53T-related synucleinopathies.

Resource utility

This unique set of lines will be an important resource to study the effects of the *SNCA* p.A53T variant in cell lines at an endogenous level. Differentiating varying types of neurons from these iPSC lines could help understand the role of cell type in selective vulnerability related to Parkinson's disease.

Resource Details

Parkinson's disease (PD) is a prevalent neurodegenerative disease characterised in part by the loss of dopaminergic neurons in substantia nigra as well as widespread pathology as the disease progresses. The motor symptomatology of the disease is associated with tremor, bradykinesia, gait and postural instability with cognitive symptoms arising later in disease (Langston, 2006).

Histopathologically, PD is typically characterised by the deposition of aggregated α-synuclein protein within surviving neurons, identified as Lewy bodies and Lewy neurites (Goedert, Jakes and Spillantini, 2017). The gene encoding *α*-synuclein protein is *SNCA* which is associated with familial and sporadic PD (Reed et al., 2019). More specifically the

first genetic variant found to segregate with PD in an autosomal dominant manner was a single base substitution, *SNCA* p.A53T (Polymeropoulos et al., 1997).

Here, we created six isogenic control lines (3 clones/line) for two iPSC lines carrying *SNCA p.*A53T from two unrelated PD patients (Table 1). We used Cas9 ribonucleoprotein (RNP) complexes with synthetic guide RNAs (crRNA/tracrRNA) to revert both p.A53T lines to WT using single stranded donor oligos (ssDO) (Table 3). After single cell cloning, two to three 96 well plates were picked per line and analysed by Sanger sequencing. Clones carrying homozygous WT nucleotides without off-target mutation in the amplicon sequence were expanded and re-sequenced to confirm target sequence (Figure 1C). Pluripotency of the lines was validated by expression of OCT4, SOX2, and NANOG by immunocytochemistry (ICC) (Figure 1B). Quantitative assessment showed that more than 98% of cells were positive for SSEA-4 in all iPSC lines (Figure 1B). All iPSC lines have a normal female karyotype (46, XX) without any obvious aberrations (Figure 1A). Short tandem repeat (STR) analysis of 16 genomic loci confirmed that the two parental iPSC lines were distinct and all reverted iPSC lines were identical to the appropriate original parent line (Supplementary File 1). The differentiation potential of the iPSC lines was confirmed by targeted differentiation into cells of all three germ layers. Additionally, ICC confirmed the expression of SOX17 (endoderm); Brachyury (mesoderm); and β-III Tubulin (ectoderm) (Figure 1D). All generated iPSC lines were free of mycoplasma contamination (Supplementary Figure 1).

In conclusion, we successfully generated three isogenic control lines for each of two iPSC lines carrying *SNCA* p.A53T to support studies of PD in human cellular models.

Materials and Methods

Growth, propagation and morphology of iPSC lines.

PPMI40760 and PPMI42072 cell lines and isogenic clones derived from these lines were grown in Gibco StemFlex media (Fisher Scientific, cat #A3349401). 10uM Rock inhibitor (STEMCELL, Cat # 72304) was used for splitting and thawing. iPSC clones were cryopreserved in Synth-A-Freeze Cryopreservation media (Thermo Scientific, Cat #A1254201).

Genome editing of iPSC lines using ribonucleoprotein.

RNP complex formation: Alt-R CRISPR-Cas9 guide RNA (crRNA) were custom designed using the IDT website https://www.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM. Alt-R CRISPR-Cas9 crRNA for A53T *SNCA* targeted region is shown in Table 3. Alt-R CRISPR-Cas9 crRNA and Alt-R CRISPR-Cas9 tracrRNA (IDT, cat #1072533) were resuspended in nuclease-free duplex buffer (IDT, cat #11010301) at 200 μM. 5 μl of 200 μM crRNA and 5 μl of 200 μM tracrRNA were mixed together, heated at 95°C for 5 minutes and then cooled to RT. Lastly, 1.7 μl (104 pmol) of Alt-R S.p. Cas9 nuclease (IDT, cat # 1081058) was mixed together with 1.2 μl (120 pmol) Alt-R CRISPR-Cas9 crRNA/tracrRNA duplex and 2.1 μl of 1xPBS solution and incubated 30 minutes at RT.

ssDO preparation: ssDO with conversion of A53T to WT (see Table 3) were synthetized by IDT and resuspended in DPBS at a concentration of 100 pmol/ µl.

Nucleofection: iPSCs were dissociated into single cells using TrypLE (Fisher Scientific, cat #A12605036) and counted. 8×10^5 cells were then pelleted at 1000 rpm for 3 minutes and subsequently gently resuspended in 100µl of P3 Primary Cell Solution from P3 Primary Cell 4D-Nucleofector X Kit L (Lonza, #V4XP-3024). Immediately prior to nucleofection, 2 µl (100 pmol/µl) of ssDO (Table 3) was added to 5µl of pre-assembled Cas9/RNP complex. 100µl of iPS cells, resuspended in P3 primary cell solution, was then combined with the Cas9/RNP complex and ssDO; thoroughly mixed and transferred to the 100 µl nucleofector cuvette (Lonza; #V4XP-3024). Immediately upon transfer, cells were transfected using the 'Primary Cell P3' program and 'CA-137' pulse code on Lonza Nucleofection machine. iPSCs were then carefully transferred using a Lonza disposable Pasteur pipette into one well of a Matrigel-coated 6-well plate containing 3 mL StemFlex media with 10 µM Rock inhibitors. Cells were cultured in a 32C/5% CO_2 incubator for two days and then transferred to a 37 C incubator. Edited iPSC pools were expanded and cryopreserved.

Generation of clones from single cell

Expanded pools were dissociated with TrypLE, counted and plated on 10cm Matrigel-coated dishes containing Stemflex media with 10 µM Rock inhibitor for two days at 10×10^3 and 15×10^3 density. Subsequently, the media was changed without Rock inhibitor for 5–7 days. Single cell colonies were expanded to 250–500 µM diameter and picked using a 100 µl pipet tip under a Bioimager microscope. Individual colonies were transferred to Matrigel-coated 96-well plates. Two to three 96 well plates were picked per line and expanded until 70–80% confluency. Each expanded plate was split into two plates: one third of the cells were transferred onto a new 96 well plate for further propagation and two thirds were used for sequencing analysis.

Sequencing

Plates with collected cells were centrifuged at 3,000 rpm for 5 minutes and resuspended in 30 µl of water. Cells were heated at 95°C for 10 minutes and 2 µl of cells were used for PCR with A53T region specific primers listed in Table 3. PCR was carried out using Terra polymerase (Takara Bio). Each PCR product was sequenced using forward and reverse primers with Applied Biosystems BigDye terminator v3.1 sequencing chemistry according to manufacturer's instructions. The products were cleaned using Agencourt CleanSEQ reagent (Beckman Coulter), run on a 3730xl DNA analyzer (Applied Biosystems, Hitachi) and analysed with Sequencher software. Positive clones were expanded and re-sequenced.

Karyotyping and STR analysis

Karyotyping and STR analysis were performed by WiCell Research Institute.

Mycoplasma detection.

Mycoplasma test was performed using PCR (ATCC, cat # 30–1012K).

Pluripotency assessment

iPSC clones were grown on Matrigel-coated coverslips, fixed with 4% PFA, and stained with pluripotency markers listed in Table 3. Images were taken on a Zeiss 880 confocal microscope. SSEA4 quantifications were performed by PE-SSEA4 antibody (Table 3). At least 50 cells per sample were counted.

Differentiation potential

iPSC clones were differentiated into three layers according to STEMdiff™ Trilineage Differentiation Kit (StemCell, Cat# 05230), fixed in 4% PFA, and stained with differentiation markers listed in Table 3. Images were taken on a Zeiss 880 confocal microscope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This research was supported by the Intramural Research Program of the NIH, National Institute on Aging and by the Michael J Fox Foundation for Parkinson's Disease Research.

References

Langston JW. The Parkinson's complex: parkinsonism is just the tip of the iceberg. Ann Neurol. 2006 Apr;59(4):591–6. doi: 10.1002/ana.20834. PMID: . [PubMed: 16566021]

Goedert M, Jakes R, Spillantini MG. The Synucleinopathies: Twenty Years On. J Parkinsons Dis. 2017;7(s1):S51–S69. doi: 10.3233/JPD-179005. PMID: ; PMCID: PMC5345650. [PubMed: 28282814]

Reed X, Bandrés-Ciga S, Blauwendraat C, Cookson MR. The role of monogenic genes in idiopathic Parkinson's disease. Neurobiol Dis. 2019 Apr;124:230–239. doi:10.1016/j.nbd.2018.11.012. Epub 2018 Nov 15. PMID: ; PMCID: PMC6363864. [PubMed: 30448284]

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science. 1997 Jun 27;276(5321):2045–7. doi: 10.1126/science.276.5321.2045. PMID: . [PubMed: 9197268]

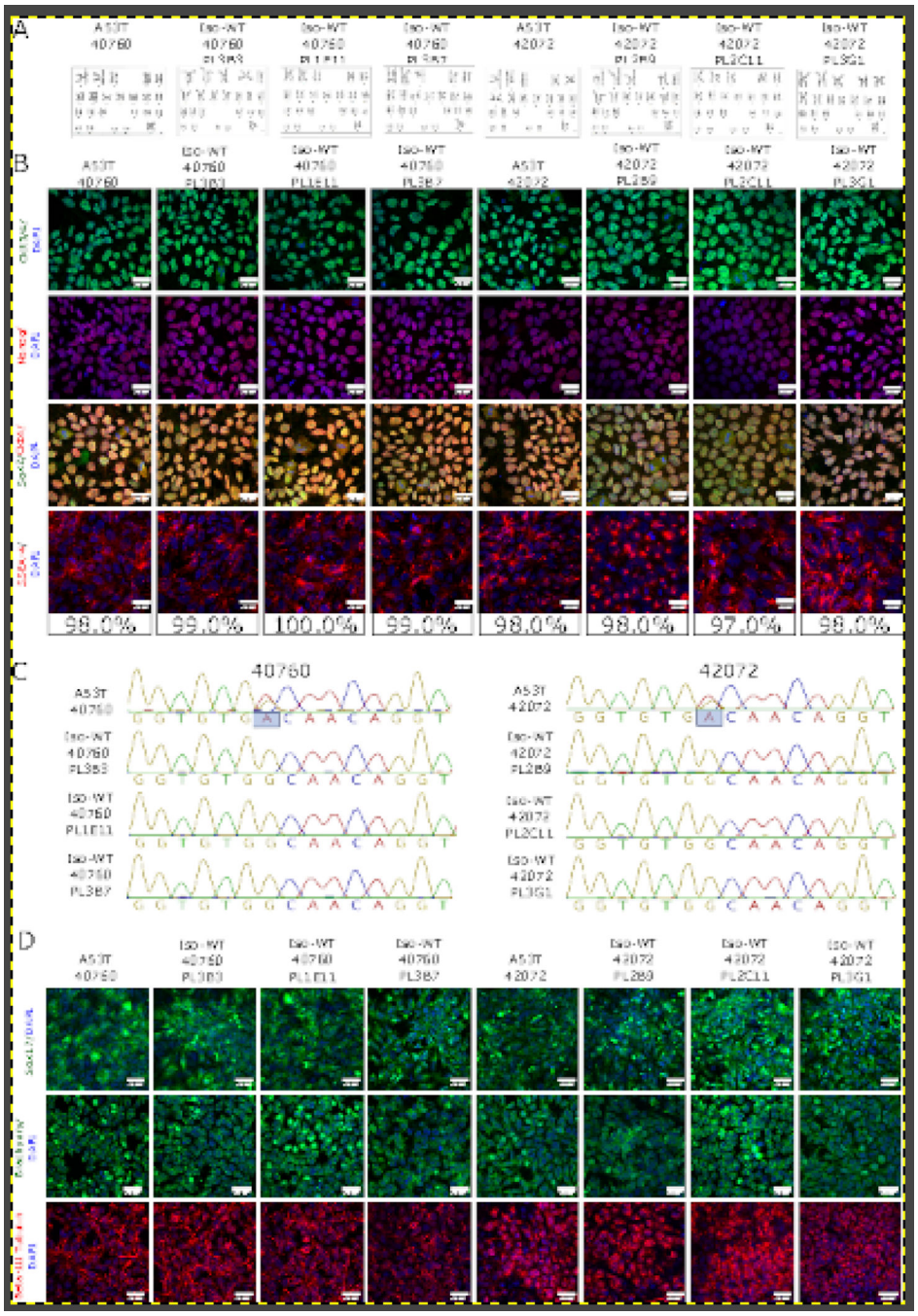

Figure 1:

Generation of SNCA A53T isogenic controls. (A) Karyotype analysis of iPS unedited lines and isogenic clones (WT = wild type, clone ID above each corresponding graph). (B) Pluripotency analysis of selected lines. Top panel - OCT 3/4 (green), second panel - NANOG (red), third panel - Sox2 (green) and Oct4 (red), and fourth panel - SSEA-4 (red). The latter allowed for quantitative percentage estimation of pluripotent cells. All lines were counterstained with nuclear stain DAPI (blue). Scale bars = 20 μm. (C) Sequencing chromatograms of SNCA A53T lines and isogenic clones. (D) ICC analysis

of differentiation potential. Endodermal layer (top panel SOX17, green); mesodermal layer (second panel, Brachuyry, green) and ectodermal layer (third panel, β-III Tubulin, red). All cells were counterstained with the nuclear dye DAPI (blue) and scale bars = 20 μm.

Table 1:

Summary of lines

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
NIAi002-A	A53T 40760	Female	N/A	N/A	*SNCA* p.A53T heterozygous variant	Parkinson disease
NIAi002-A-1	Iso-WT 40760 PL1E11	Female	N/A	N/A	WT *SNCA* gene sequence	N/A
NIAi002-A-2	Iso-WT 40760 PL3B3	Female	N/A	N/A	WT *SNCA* gene sequence	N/A
NIAi002-A-3	Iso-WT 40760 PL3B7	Female	N/A	N/A	WT *SNCA* gene sequence	N/A
NIAi003-A	A53T 42072	Female	N/A	N/A	*SNCA* p.A53T heterozygous variant	Parkinson disease
NIAi003-A-1	Iso-WT 40760 PL2B9	Female	N/A	N/A	WT *SNCA* gene sequence	N/A
NIAi003-A-2	Iso-WT 40760 PL2C11	Female	N/A	N/A	WT *SNCA* gene sequence	N/A
NIAi003-A-3	Iso-WT 40760 PL3G1	Female	N/A	N/A	WT *SNCA* gene sequence	N/A

Table 2:

Characterization and validation

Classification	Test	Result	Data
Morphology	Photography	All iPSC lines are morphologically normal	Not shown; available from authors.
Phenotype	Qualitative analysis	Confirmed expression of pluripotency markers: OCT4, NANOG, and SOX2	Figure 1, panel B
	Quantitative analysis	Assessed % of positive cells for pluripotency cell surface marker, SSEA-4. All lines are more than 98% positive.	Figure 1 panel B
Genotype	Karyotype (G-banding) and resolution	1. 46XX, Resolution 425–450 2. 46XX, Resolution 425–525 3. 46XX, Resolution 400–425 4. 46XX, Resolution 425–450 5. 46XX, Resolution 375–475 6. 46XX, Resolution 400–450 7. 46XX, Resolution 400–450 8. 46XX, Resolution 450–475	Figure 1 panel A
Identity	Microsatellite PCR (mPCR) OR STR analysis	STR analysis	Supplementary data 1
		16 specific loci were tested. All isogenic lines matched 100%.	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	1. A53T 40760 heterozygous	Figure 1 panel C
		2. Iso-WT 40760 PL3B3 3. Iso-WT 40760 PL1E11 4. Iso-WT 40760 PL3B7 5. A53T 42072 heterozygous 5. Iso-WT 42072 PL2B9 6. Iso-WT 42072 PL2C11 7. Iso-WT 42072 PL3G1	
	Southern Blot OR WGS	Not performed	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing was done by RT-PCR. All clones-negative	Supplementary figure 1
Differentiation potential	e.g. Embryoid body formation OR Teratoma formation OR Scorecard OR Directed differentiation	The STEMdiff™ Trilineage Differentiation Kit (StemCell) was used to test differentiation potential. We confirmed the expression of endoderm (SOX-17), mesoderm (Brachyury) and ectoderm (-Tubulin III) markers in all clones.	Figure 1 panel D
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	N/A
	HLA tissue typing	Not performed	N/A

Table 3:

Reagents details

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	Mouse anti-SOX2	1:200	Millipore, Cat# MAB4423, RRID:AB_11213224
Pluripotency Markers	PE anti-human SSEA-4	1:200	BioLegend Cat# 330406, RRID:AB_1089206
Pluripotency Markers	Mouse anti-OCT3/4	1:200	Santa Cruz Biotechnology Cat# sc5279, RRID: AB_628051
Pluripotency Markers	Rabbit anti-OCT4	1:200	Abcam Cat #ab19557, RRID:N/A
Pluripotency Markers	Mouse anti-Nanog	1:200	Millipore Cat# MABD24, RRID:AB_11203826
Differentiation Markers	Goat anti-SOX17	1:200	R and D Systems Cat# AF1924, RRID:AB_355060
Differentiation Markers	Goat anti-Brachyury	1:200	R and D Systems Cat# AF2085, RRID:AB_2200235
Differentiation Markers	Chicken anti-beta-III Tubulin	1:200	Novus Cat# NB100–1672, RRID:AB_522025
Secondary antibodies	Donkey anti-mouse Alexa Fluor 488	1:500	Thermo Fisher Scientific Cat# A21202, RRID:AB_141607
Secondary antibodies	Donkey anti-mouse Alexa Fluor 568	1:500	Thermo Fisher Scientific Cat# A10037, RRID:AB_2534013
Secondary antibodies	Donkey anti-rabbit Alexa Fluor 568	1:500	Thermo Fisher Scientific Cat# A10042, RRID:AB_2534017
Secondary antibodies	Donkey anti-goat Alexa Fluor 488	1:500	Thermo Fisher Scientific Cat# A11055, RRID:AB_2534102
Secondary antibodies	Goat anti-chicken Alexa Fluor 568	1:500	Thermo Fisher Scientific Cat# A11041, RRID:AB_2534098
Primers			
	Target	**Forward/Reverse primer (5′–3′)**	
Targeted variant analysis/sequencing	A53T	CTAGCTAATCAGCAATTTAAGGCTA/GCTC AGTGATTGTTTTACAATTTCA	
CRISPR reagents			
Targeted mutation	**crRNA used for Cas9 editing**	**Donor oligo sequence**	
A53T	GTGGTGCATGGTGTGACAAC agg	AAAACTAGCTAATCAGCAATTTAAGGCTAGCTT GAGACTTATGTCTTGAATTTGTTTTTGTAGGCT CCAAAACCAAGGAGGGAGTGGTGCATGGTGT G**G**CAACAGGTAAGCTCCATTGTGCTTATATCC	
		AAAGATGATATTTAAAGTATCTAGTGATTAGTG TGGCCCAGTATTCAAGATTCCTATGAAATTGTAAAAC	

Resource Table:

Unique stem cell lines identifier	NIAi002-A NIAi002-A-1 NIAi002-A-2 NIAi002-A-3 NIAi003-A NIAi003-A-1 NIAi003-A-2 NIAi003-A-3
Alternative names of stem cell lines	A53T SNCA 40760 (NIAi002-A) Iso-WT SNCA 40760 PL1E11 (NIAi002-A-1) Iso-WT SNCA 40760 PL3B3 (NIAi002-A-2) Iso-WT SNCA 40760 PL3B7 (NIAi002-A-3) A53T SNCA 42720 (NIAi003-A) Iso-WT SNCA 42720 PL2B9 (NIAi003-A-1) Iso-WT SNCA 42072 PL2C11 (NIAi003-A-2) Iso-WT SNCA 42072 PL3G1 (NIAi003-A-3)
Institution	National Institutes of Health, National Institute on Aging
Contact information of distributor	Mark R Cookson: cookson@mail.nih.gov
Type of cell lines	iPSC
Origin	Human
Cell Source	The Michael J. Fox Foundation for Parkinson's Research (MJFF), Parkinson's Progression Markers Initiative (PPMI).
Clonality	Clonal
Method of reprogramming	Sendai virus
Multiline rationale	Isogenic clones from two unrelated donors carrying the same pathogenic variant in a PD related gene
Gene modification	Yes
Type of modification	Revert *SNCA* p.A53T to WT sequence
Associated disease	Parkinson's disease
Gene/locus	SNCA/PARK1
Method of modification	RNP CRISPR/Cas9
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	N/A
Cell line repository/bank	Indiana University https://hpscreg.eu/cell-line/NIAi002-A https://hpscreg.eu/cell-line/NIAi002-A-1 https://hpscreg.eu/cell-line/NIAi002-A-2 https://hpscreg.eu/cell-line/NIAi002-A-3 https://hpscreg.eu/cell-line/NIAi003-A https://hpscreg.eu/cell-line/NIAi003-A-1 https://hpscreg.eu/cell-line/NIAi003-A-2 https://hpscreg.eu/cell-line/NIAi003-A-3

Ethical approval	Original line obtained from MJFF. Ethical licence/Sponsor Protocol number: 001.