Title: Generation of PRKN and PINK1-KO and double KO cell lines from healthy induced pluripotent stem cells using CRISPR/Cas9 editing

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Abstract:

Autosomal recessive mutations in either *PRKN* or *PINK1* are associated with early-onset Parkinson's disease. The corresponding proteins, PRKN, an E3 ubiquitin ligase, and the mitochondrial serine/threonine-protein kinase PINK1 play a role in mitochondrial quality control. Using CRISPR/CAS9 technology we generated three human iPSC lines from the well characterized AIW002-02 control line. These isogenic iPSCs contain homozygous knockouts of *PRKN* (PRKN-KO, CBIGi001-A-1), *PINK1* (PINK1-KO, CBIGi001-A-2) or both *PINK1* and *PRKN* (PINK1-KO/PRKN-KO, CBIGi001-A-3). The knockout lines display normal karyotypes, express pluripotency markers and upon differentiation into relevant brain cells or midbrain organoids may be valuable tools to model Parkinson's disease.

1. Resource Table:

Unique stem cell lines identifier	CBIGi001-A-1 CBIGi001-A-2 CBIGi001-A-3
Alternative name(s) of stem cell lines	PRKN-KO/AIW002-02 (PRKN-KO) PINK1-KO/AIW002-02 (PINK1-KO) PINK1-KO/PRKN-KO/AIW002-02 (PINK1-KO/PRKN-KO)
Institution	The Montreal Neurological Institute (The Neuro)
Contact information of the reported cell line distributor	Thomas Durcan, PhD; thomas.durcan@mcgill.ca
Type of cell lines	iPSC
Origin	Human
Additional origin info	Age: 37 Sex: Male Ethnicity: Caucasian
Cell Source	AIW002-02 (CBIGi001-A)
Clonality	Clonal
Method of reprogramming	Parental AIW002-02 iPSCs were derived from PBMCs of a healthy male using a four factor (Klf4, Oct3/4, Sox2, cMyc) Sendai virus-based system

Multiline rationale	Isogenic genome-edited disease cell lines
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	N/A
Cell culture system used	mTESR1 medium on Matrigel coated plates
Type of Genetic Modification	Homozygous gene knockout
Associated disease	Parkinson's disease
Gene/locus	<i>PRKN</i> (6q25.2-q27) (77 bp deletion in Exon 2) <i>PINK1</i> (1p35-p36) (17 bp deletion/18 bp “Stop-tag” insertion) <i>PINK1/PRKN</i> (PRKN-KO: 77 bp deletion in exon 2)/ <i>PINK1</i> -KO: 5 bp deletion in Exon 1)
Method of modification/site-specific nuclease used	CRISPR/Cas9-mediated gene knockout
Site-specific nuclease (SSN) delivery method	PRKN-KO: Plasmid transfection <i>PINK1</i> -KO and <i>PINK1</i> -KO/ <i>PRKN</i> -KO: Nucleofection
All genetic material introduced into the cells	PRKN-KO: CRISPR/Cas9 plasmid
Analysis of the nuclease-targeted allele status	Sequencing of targeted alleles
Method of the off-target nuclease activity surveillance	Targeted PCR/sequencing
Name of transgene	N/A
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	PRKN-KO: puromycin
Inducible/constitutive system details	N/A
Date archived/stock date	2021-01-22
Cell line repository/bank	https://hpscreg.eu/cell-line/CBIGi001-A-1 https://hpscreg.eu/cell-line/CBIGi001-A-2 https://hpscreg.eu/cell-line/CBIGi001-A-3
Ethical/GMO work approvals	McGill University Health Centre Research Ethics Board (DURCAN_IPSC/2019-5374)
Addgene/public access repository recombinant DNA sources’ disclaimers (if applicable)	pSpCas9(BB)-2A-Puro (PX459) was a gift from Feng Zhang (Addgene plasmid # 48139; http://n2t.net/addgene:48139) RRID: Addgene_48139

Resource utility

PRKN and PINK1 are well known Parkinson’s disease-associated genes. We generated isogenic PRKN-KO, PINK1-KO, and double PINK1-KO/PRKN-KO human iPSC lines, allowing differentiation into neuronal and glial cells for studying Parkinson’s disease and related neurodegenerative disorders. These lines should accelerate understanding of PRKN and PINK1-mediated immune and mitochondrial quality control pathways.

Resource Details

Two Parkinson’s disease-associated genes, PRKN and PINK1, encode Parkin, a E3-ubiquitin ligase, and the mitochondrial kinase PINK1, proteins critical for mitochondrial quality control (Durcan and Fon, 2015; Sison et al., 2018). We derived PRKN, PINK1 and PINK1/PRKN knockout (KO) lines from AIW002-02 control iPSCs reprogrammed from a 37-year-old healthy male (Chen et al., 2021). Guide RNAs targeting PRKN were cloned into the PX459 vector and transfected into parental AIW002-02 iPSCs. After selection and PCR screening,

Sanger sequencing showed one clone contained a homozygous 77 bp deletion in *PRKN* (Fig. 1A), resulting in a frameshift mutation. To knock out PINK1 expression, AIW002-02 or PRKN-KO iPSCs were nucleofected with a combination of CAS9 protein, a sgRNA targeting PINK1 Exon1 (3' of the ATG start codon), and a ssODN containing a "Stop-tag". Following limiting dilution, PINK1-KO clones were identified by digital droplet PCR and Sanger sequencing (Fig. 1A), confirming replacement of 17 bp in the PINK1 Exon 1 with a 18 bp sequence containing a stop codons in three reading frames (blue). In the double PINK1-KO/PINK1-KO, however, a homozygous five bp deletion, leading to a frameshift was found (red). Following CRISPR gene KO, qPCR analysis revealed PRKN mRNA expression was < 15% of the expression observed in the parental AIW002-02 iPSCs. In the two PINK1 KO lines, PINK1 expression was also less than 14% of AIW002-02 (Supplementary Fig. 1D). PCR and Sanger sequencing of the top five potential genome-wide off-target sites showed the KO lines lacked off-target mutations or PX459 sequences (Supplementary Fig. 1A, C). Short tandem repeat (STR) profiling demonstrated all KO clones were derived from the AIW002-02 parental line (available from the authors).

The PRKN-KO, PINK1-KO and PINK1-KO/PRKN-KO iPSCs showed typical human embryonic stem cell-like morphology by phase-contrast microscopy (Fig. 1B), were ≥99.8% positive for the pluripotency marker SSEA-4 and 100% positive for the iPSC marker CD200 by flow cytometry (Fig. 1C). Immunocytochemistry imaging confirmed all KO iPSCs expressed the pluripotency markers NANOG, TRA1-60, SSEA-4, and OCT3/4 (Fig. 1D). Normal karyotypes were observed for the new KO lines (Fig. 1E) and they did not contain any of the eight most common of karyotypic abnormalities reported in human iPSCs (Supplementary Fig. 1E). All three KO lines were mycoplasma negative as determined by a luminescence-based assay (Supplementary Fig. 1B). Following embryoid body formation and *in vitro* differentiation, immunocytochemistry for ectoderm (nestin), mesoderm (SMA), and endoderm (vimentin) markers demonstrated that PRKN-KO, PINK1-KO and PINK1-KO/PRKN-KO lines were able to form each of the three germ layers. Trilineage differentiation followed by qPCR showed significantly increased expression (lower C_q values) of the ectoderm (*PAX6*), endoderm (*FOXA2*) and mesoderm (*ACTA2*, *MIXL1*) markers (Fig. 1G) compared to iPSCs. In addition, using established protocols (Chen et al., 2019), neuronal precursors from all KO lines were differentiated into tyrosine hydroxylase (TH), MAP2, Tuj1 positive neurons (Supplementary Fig. 1F), indicating that dopaminergic neurons can be generated from these KO iPSCs. These well-characterized isogenic iPSC knockout lines will serve as *in vitro* models for studying PRKN and PINK1-mediated pathways in Parkinson's disease.

Materials and Methods

iPSC culture

AIW002-02 iPSCs were reprogrammed and characterized as described (Chen et al., 2021). iPSCs were maintained in mTeSR1 media (Stemcell Technologies) on Matrigel (Corning)-coated plates at 37°C with daily media changes, and passaged when 70-80% confluent with Gentle Cell Dissociation Reagent (Stemcell).

CRISPR/Cas9 knockout

AIW002-02 iPSCs (80,000/well/24-well plate, seeded 24 hours previously) were co-transfected (~0.5 µg/vector) using Lipofectomine™ Stem Transfection Reagent (ThermoFisher Scientific). Twenty-four hours later, cells were selected in puromycin (0.3 µg/ml) for 48h and grown until single clones formed. KO clones were verified by PCR with PRKN-specific primers (Table 2) and Sanger sequencing.

Alternatively, ribonucleoprotein complexes containing CAS9 protein (1 µl; stock 61 µM), PINK1 sgRNA (3 µl; stock 100 µM) and ssODN (1 µl, stock 100 µM) in 20 µl of nucleofection buffer P3 were nucleofected (program CA137, 4D-Nucleofector Device, Lonza) into 500,000 detached iPSCs (Deneault et al., 2021). After limiting dilution, KO clones were identified by ddPCR (QX200™ Droplet Reader, Bio-Rad) and Sanger sequencing.

Flow cytometry

iPSCs were detached using TrypLE Express (ThermoFisher Scientific), stained with Live Dead Aqua (Invitrogen) for viability, then blocked with Human TruStain FcX (Biolegend). Cells were stained with FACS antibodies (Table 2) for 30 min at RT, before data acquisition on an Attune NxT Flow Cytometer (ThermoFisher Scientific) and FlowJo analysis.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde at RT for 20 mins, permeabilized with 0.2% Triton X-100/PBS for 10 min, blocked in 5% donkey serum/1% BSA/0.05% Triton X-100/PBS for 2 h, incubated with primary antibodies (Table 2) in blocking buffer overnight at 4°C, secondary antibodies (Table 2) for 2 h at RT, then counterstained with Hoechst 33342 for 5 min. Images were acquired using the automated Evos FL-Auto2 imaging system (ThermoFisher Scientific).

Karyotyping and genomic integrity analysis

G-band karyotyping was performed by Wicell Cytogenetics, Madison, WI. Genomic integrity was analyzed using DNA extracted from iPSCs with a Genomic DNA Mini Kit (Geneaid) and the hPSC Genetic Analysis Kit (Stemcell).

Three Germ Layer Differentiation

Embryoid bodies (EBs) were generated from iPSCs, allowed to spontaneously differentiate, fixed, and analyzed by immunocytochemistry. Pluripotency was also assessed with The STEMdiff Trilineage differentiation kit (Stemcell) (Chen et al., 2021).

qPCR analysis

cDNA was generated (iScript Reverse Transcription Supermix; BioRad) from purified RNA (NucleoSpin RNA kit; Takara). Raw data from the QuantStudio 5 Real-Time PCR System (Applied Biosystems) were processed with the Auto-qPCR app using GADPH or ACTB expression as endogenous controls (Chen et al., 2021).

Dopaminergic neuron differentiation

iPSCs were induced into DA-NPCs according to established protocols (Chen et al., 2019). DA-NPCs were differentiated into neurons in neurobasal medium, supplemented with N2 and B27, 1 µg/ml laminin, 500 µM db-cAMP, 20 ng/ml BDNF, 20 ng/ml GDNF, 200 µM ascorbic acid, 100 nM Compound A and 1 ng/ml TGF-β. Immunostaining was conducted at 4 weeks maturation.

Short Tandem Repeat Analysis

STR analysis was performed with the GenePrint® 10 System (Promega) at The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, ON.

Mycoplasma

iPSC culture media were tested using the luminescence-based MycoAlert Mycoplasma Detection kit (Lonza).

Declaration of Competing Interest

The authors declare no competing interests.

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Fig. 1. Characterization of isogenic PRKN-KO, PINK1-KO, and PINK1-KO/PRKN-KO iPSC lines.

Appendix A. Supplementary data

Supplementary Fig. 1

Table 1: Characterization and validation

Classification (optional <i>italicized</i>)	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel B
Pluripotency status evidence for the described cell line	Qualitative analysis (Immunocytochemistry)	Positive expression of pluripotency markers NANOG, Tra1-60 SSEA-4 and OCT3/4	Fig. 1 panel D
	Quantitative analysis (Flow cytometry)	PRKN-KO (SSEA-4: 99.9%, CD200: 100%); PINK1-KO (SSEA-4: 99.9%, CD200: 100%); PINK1-KO/PRKN-KO (SSEA-4: 99.8%, CD200: 100%)	Fig. 1 panel C
Karyotype	Karyotype (G-banding) and resolution	46,XY Resolution 450–600	Fig. 1 panel E
	Chromosomal abnormality analysis (qPCR-based)	Normal	Supplementary Fig.1 panel E
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site or targeted allele-specific PCR	PCR + sequencing, homozygous	Fig. 1 panel A
	Transgene-specific PCR	N/A	N/A
Verification of the absence of random plasmid integration events	PCR	Absence of pSpCas9(BB)-2A-Puro (PX459) integration in PRKN-KO	Supplementary Fig.1 panel C
Parental and modified cell line genetic identity evidence	STR analysis	10 specific loci were tested. All isogenic lines matched 100%	Supplementary data 1 Submitted in archive with journal
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR or RT-PCR product)	PRKN-KO: Homozygous, 77 bp deletion in <i>PRKN</i> PINK1-KO: Homozygous, 18 bp indel in <i>PINK1</i> PINK1-KO/PRKN-KO: Homozygous, 5 bp deletion in <i>PINK1</i>	Fig. 1 panel A
	PCR-based analyses (qPCR)	Decreased expression of PRKN and/or PINK1 in KO lines	Supplementary Fig.1 panel D
	Southern Blot or WGS; western blotting (for knock-outs, KOs)	Not performed	N/A

Off-target nuclease analysis	PCR across top 5 predicted top likely off-target sites	Lack of NHEJ-caused mutagenesis in the top predicted off-target Cas nuclease activity	Supplementary Fig.1 panel A
Specific pathogen-free status	Mycoplasma	Micoplasma testing by luminescence. Negative	Supplementary Fig. 1 panel B
Multilineage differentiation potential	Embryoid body formation (Immunocytochemistry)	Proof of differentiation into three germ layers by immunostaining for Nestin , SMA, and Vimentin	Fig. 1 panel F
	Directed differentiation mRNA expression (qPCR)	Increased expression of <i>PAX6</i> (ectoderm), <i>MIXL1</i> (mesoderm), <i>FOXA2 and Vimentin</i> (endoderm)	Fig. 1 panel G
	Directed differentiation (Immunocytochemistry)	Dopaminergic neuron markers (TH, MAP2, Tuj)	Supplementary Fig.1 panel F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Negative	Available from the authors
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping	Not performed	N/A
	HLA tissue typing	Not performed	N/A

Table 2: Reagents details

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit anti-Nanog	1:200	Abcam Cat# ab21624, RRID:AB_446437
	Mouse anti-TRA1-60	1:200	Stemcell Technologies Cat# 60064, RRID:AB_2686905
	Mouse anti-SSEA-4	1:500	Santa Cruz Biotechnology Cat# sc-21704, RRID:AB_628289
	Goat anti-OCT3/4	1:1000	Santa Cruz Biotechnology Cat# sc-8628, RRID:AB_653551
Differentiation Markers	Rabbit anti-Nestin	1:500	Abcam Cat#ab92391, RRID:AB_10561437
	Rabbit anti-SMA1	1:200	Abcam Cat# ab5694, RRID:AB_2223021
	Rabbit anti-Vimentin	1:200	Abcam Cat#ab92547, RRID:AB_10562134
	Rabbit anti-TH	1:500	Pel-Freez Biologicals Cat# P40101-

			150, RRID:AB_2617184
	Mouse anti-Tubulin-βIII	1:1000	Millipore Cat# MAB5564, RRID:AB_11212768
	Chicken anti-MAP2	1:1000	EnCor Biotechnology Cat# CPCA-MAP2; RRID: AB_2138173
Flow cytometry antibodies	PE anti-human CD200	1:20	BioLegend Cat# 399803, RRID:AB_2861015
	APC anti-human SSEA-4	1:20	BioLegend Cat#330417, RRID:AB_2616818
Secondary antibodies	Dylight 488 donkey anti-mouse IgG	1:5000	Abcam Cat# ab96875, RRID:AB_10698084
	Dylight 488 donkey anti-rabbit IgG	1:500	Abcam Cat# ab96891, RRID:AB_10679664
	Dylight 550 donkey anti-mouse IgG	1:500	Abcam Cat# ab96876, RRID: AB_10679663
	Dylight 650 donkey anti-mouse IgG	1:500	Abcam Cat# ab96878, RRID:AB_10680869
	Dylight 650 donkey anti-rabbit IgG	1:500	Abcam Cat# ab96894, RRID:AB_10680167
	AlexaFluor647 donkey anti-goat	1:500	Molecular Probes Cat# A-21447, RRID:AB_141844
	Dylight 650 goat anti-chicken IgY	1:500	Abcam Cat# ab96950, RRID:AB-10679408
Nuclear stain	Hoechst 33342 (HO342)	1:3000	ThermoFisher Scientific Cat # H3570
Site-specific nuclease			
Nuclease information	CRISPR/Cas9		
Delivery method	PRKN-KO PINK1-KO PINK1-KO/PRKN-KO	Plasmid transfection Nucleofection Nucleofection	
Selection/enrichment strategy	PRKN-KO PINK1-KO PINK1-KO/PRKN-KO	Puromycin selection Limiting dilution/ddPCR Limiting dilution/ddPCR	
Probes for qPCR			
	Target	Assay ID (TaqMan)	
Pluripotency Markers	POU5F1(OCT3/4)	Hs04260367_gH	
Mesoderm Markers	MIXL1	Hs00430824_g1	
	ACTA2	Hs00426835_g1	
Endoderm Markers	FOXA2	Hs05036278_s1	
	VIM	Hs00958111_m1	
Ectoderm Markers	PAX6	Hs01088114_m1	
	NCAM1	Hs00941830_m1	
House Keeping	GADPH	Hs02786624_g1	
	ACTB	Hs01060665_g1	
Targeted knockout mRNA expression	PRKN	Hs01038318_m1	
	PINK1	Hs00260868_m1	
Primers and Oligonucleotides used in this study			
	Target	Forward/Reverse primer (5′-3′)	
PCR to detect integration of Cas9 vectors	pSpCas9(BB)-2A-Puro (PX459)	AGAACCTGCCCAACGAGAAG/ CGAACAGGTGGGCATAGGTT	
PRKN gRNA1	PRKN exon 2	CTCCAGCCATGGTTTCCAG	

PRKN gRNA2	PRKN exon 2	CTGCGAAAATCACACGCAAC
PINK1 gRNA	PINK1 exon 1	ACTGGCGGAAGAAGCGGAGA
PINK1 Stop-tag ssODN	PINK1 exon 1	CTGGGCCGAGGACCGGGCGGGAGCCTCGCAGGG TCGGGCTCGGGCTCCCTAACCGTCTGCCGGCGTAACT AGCTGACGGTGGCCGGGCTGGCGGCGCGGTTGCAG CGGCAGTTCGTGGTGGGGCCTGGG
Targeted mutation analysis/sequencing	PRKN PCR PF1/PF2	AAGGGCTTCGAGTGATGCTC/ CCTTGCTGCTCCTGTAGTCA
	PRKN sequencing	CCTTGCTGCTCCTGTAGTCA
	PINK1 PCR PF1/PF2	TCACAAGACCTCGAATGCTG/ GCTCACCTGGATCTCCTGAC
	PINK1 sequencing	TAGCCCGAAGGCCAGAA
Droplet digital PCR	PINK1 PCR PF1/PF2	GGGCTCCCTAACCGTCT/ TAGCCCGAAGGCCAGAA
	WT probe Stop-tag probe	/5HEX/CGCTT+CT+TC+C+GC/3IABkFQ/ /56-FAM/CGTAA+C+TA+G+C+T+GA/3IABkFQ
Top off-target mutagenesis predicted site sequencing primers	PRKN OT1-F&R	AAGCCAAGCTGGGCAAGTAT/ ATACATGCCACCTGCAACCT
	PRKN OT2-F&R	TGTGTGAAAGGGACAGGGTG/ CCTGACATTCCGAATCCAAAGC
	PRKN OT3-F&R	TCGTCATGGCTCAAGTGCTG/ CATCTTTCTGTGGCCCAAGC
	PRKN OT4-F&R	GCTGAGTTGGGAGGATCACC/ GAAAGAGACCTTCAGCCCCC
	PRKN OT5-F&R	CTGAGAGGTTGGAGCAGAAGA/ ACACGCATTTTTCCCTATCCCT
	PINK1 OT1-F&R	CAGCAGGAGACCTTCAAGCA/ AAAACAGTGGCACCAAGGA
	PINK1 OT2-F&R	AGAGCAGGAGTATTGGGGAGT/ AGGTCTCCCCACTTCTCTGG
	PINK1 OT3-F&R	TTCTGGCACCCCTGTCTCTA/ GTTTCAGGCCAACCTCCTCA
	PINK1 OT4-F&R	GCACTGTTCTTTTCCAGGC/ CCCACCAGATGCCGTTTATA
	PINK1 OT5-F&R	CTTCAGTCACACAGCCTCC/ CCCTGCTTCCTCGCTAAACA