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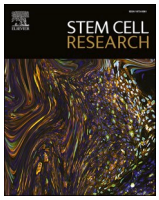

Lab Resource: Genetically-Modified Multiple Cell Lines

Generation of two edited iPSCs lines by CRISPR/Cas9 with point mutations in PKP2 gene for arrhythmogenic cardiomyopathy in vitro modeling

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A B S T R A C T

The arrhythmogenic cardiomyopathy (ACM) is an inherited heart muscle disease characterized by the progressive replacement of contractile myocardium by fibro-fatty adipose tissue, that generates ventricular arrhythmias and sudden death in patients. The ACM has a genetic origin with alterations in desmosomal genes with the most commonly mutated being the *PKP2* gene. We generated two CRISPR/Cas9 edited iPSCs lines, one iPSC line with a point mutation in PKP2 reported in patients with ACM and another iPSC line with a premature stop codon to knock-out the same gene.

1. Resource Table

Unique stem cell line identifier	INEUi002-A-1 INEUi002-A-2
Alternative name(s) of stem cell line	PKP2 S140F (INEUi002-A-1) PKP2 KO (INEUi002-A-2)
Institution	Fundación para la Lucha contra las Enfermedades Neurológicas de la Infancia (FLENI). LIAN-INEU
Contact information of the reported cell line distributor	Dr. Santiago Miriuka. smiriuka@fleni.org.ar/Dra. Lucia N. Moro lmoro@fleni.org.ar
Type of cell line	iPSC
Origin	Human
Additional origin info	N/A
Cell Source	iPSCs
Method of reprogramming	N/A
Clonality	Clonal isolation approach
Evidence of the reprogramming transgene loss	N/A
The cell culture system used	ACF conditions
Type of the Genetic Modification	Induced mutation
Associated disease	Arrhythmogenic Cardiomyopathy
Gene/locus	NM_001005242.3 (PKP2): c.419C > T (p.Ser140Phe) NM_001005242.3 (PKP2): c.422 + C (p.153X)
Method of modification/user-customisable nuclease (UCN) used,	CRISPR/Cas9

(*continued on next column*)

(*continued*)

the resource used for design optimisation	
User-customisable nuclease (UCN) delivery method	Encoding plasmid transfection
All double-stranded DNA genetic material molecules introduced into the cells	Cas9 plasmid, HDR donor ssODN
Analysis of the nuclease-targeted allele status	Sequencing of the targeted allele by Sanger sequencing
Method of the off-target nuclease activity prediction and surveillance	We used the Benchling software prediction for possible off targets and determined the sequences of the top three.
Descriptive name of the transgene	N/A
Eukaryotic selective agent resistance cassettes	N/A
Inducible/constitutive expression system details	N/A
Date archived/stock creation date	04/17/2023
Cell line repository/bank	INEUi002-A-1: https://hpscreg.eu/cell-line/INEUi002-A-1 INEUi002-A-2: https://hpscreg.eu/cell-line/INEUi002-A-2
Ethical/GMO work approvals	The FLENI Ethics Committee approved all animal and human procedures. The work described was carried out in accordance with The Code of Ethics of theWorld Medical Association.
Addgene/public access repository recombinant DNA sources	pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene ID: 62988)

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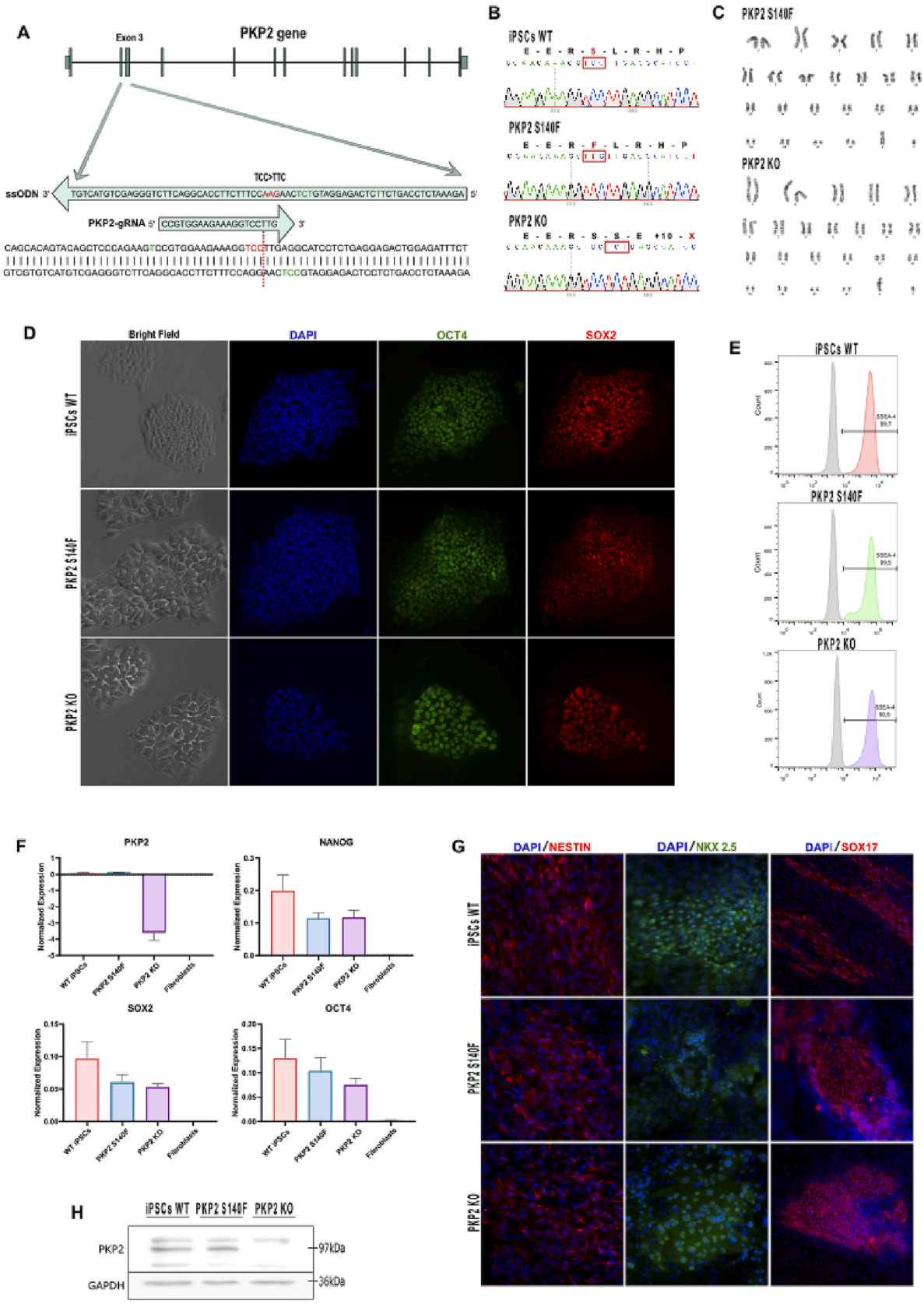

Fig. 1. Characterization of PKP2 KO and PKP S140F cell lines.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Typical induced pluripotent stem cell morphology	Fig. 1 Panel D
Pluripotency status evidence for the described cell line	Immunocytochemistry	Positive expression of pluripotency markers OCT3/4 and SOX2	Fig. 1 panel D
	Flow cytometry and RT-qPCR	Flow cytometry for SSEA-4 PKP2 S140F: 99.4% PKP2 KO: 99.9% Expression of OCT4, SOX2 and NANOG by RT-qPCR	Fig. 1 panel E-F
Karyotype	Karyotype (G-banding and resolution)	PKP2 S140F: 46, XY PKP2 KO: 46, XY	Fig. 1 panel C
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site	PKP2 S140F: PCR + sequencing, obtaining the desired edition (c.419C > T) PKP2 KO: PCR + Sanger sequencing, obtaining an addition of a "C" in c.420 (c.422 + C)	Fig. 1 panel B
	Evaluation of the homozygous status of introduced genomic alteration by Sanger sequencing and quantitative genotyping PCR (gqPCR)	PKP2 S140F and PKP2 KO are homozygous for the desire mutation	Fig. 1 panel B Supplementary Fig. E
	Transgene-specific PCR	N/A	N/A
Verification of the absence of random plasmid integration events	PCR	PCR detection for plasmid backbones	Supplementary Fig. D
Parental and modified cell line genetic identity evidence	STR analysis	DNA Profiling. Both iPSC clonal cell lines matched to the individual host profile. 27 loci were analyzed.	Supplementary File
Mutagenesis/genetic modification outcome analysis	Sequencing	Confirmation of the precise nature of introduced alteration	Fig. 1 panel B
	PCR-based analyses		Supplementary Fig. C
	Western Blotting (for knock-out)	Demonstration of protein elimination in KO.	Fig. 1 panel H
Off-target nuclease activity analysis	PCR across top predicted top likely off-target sites	The top predicted off-target of gRNA were not altered after CRISPR/Cas9 transfection.	Supplementary Fig. A
Specific pathogen-free status	Mycoplasma	Mycoplasma testing by PCR: Negative	Supplementary Fig. B
Multilineage differentiation potential	Embryoid body formation	Both iPCS lines were differentiated into cells of three germ layers, demonstrated by expression of Nestin (ectoderm), TBX6 (mesoderm) and SOX17 (endoderm).	Fig. 1 panel G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping HLA tissue typing	N/A N/A	N/A N/A

2. Resource utility

Dozens of gene mutations have been reported for the ACM. However, it is not possible to model in-vitro all the ACM variants using patient samples for therapy discovery. Considering this, we aim to generate iPSC lines with point mutations reported in ACM patients using the CRISPR/Cas9 gene editing system.

3. Resource details

The arrhythmogenic cardiomyopathy (ACM) is an inherited heart muscle disease characterized by the progressive replacement of contractile myocardium by fibro-fatty adipose tissue. ACM patients often have ventricular arrhythmias, heart failure and eventually sudden death (Saffitz et al., 2010). The ACM has a genetic origin with mutations in genes involved in different cellular functions, including desmosome structure, ion channels, cytoskeleton organization, calcium regulation and sarcomere formation. Mutations in the desmosomal genes (*PKP2, PKG, DSP, DSG-2* and *DSC-2*) are the most often found in patients with ACM, with PKP2 being the most common gene associated with the disease (Gerull et al., 2004). PKP2 plays a fundamental role in maintaining the structural integrity of cardiomyocytes. It has been reported that PKP2 knock out (KO) mice are non-viable due to reduced trabeculation, cytoskeletal disarray and cardiac wall rupture (Grossmann et al., 2004). There are a large number of mutational variants reported for the ACM, making it difficult to encompass the different genotypes to model the disease using patient-derived iPSCs. In order to overcome this limitation, iPSCs lines can be generated specifically with the mutations reported in ACM patients using gene editing. The aim of this work was to generate

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers (Immunofluorescence)	Mouse anti-OCT4	1:50	Thermo Fisher Scientific Cat# MA1-104, RRID:AB_2536771
	Rabbit anti SOX2	1:200	Cell Signaling Technology Cat# 3579, RRID:AB_2195767
Pluripotency Markers (Flow cytometry)	SSEA-4 Antibody, Alexa Fluor; 488 conjugate	1:50	Molecular Probes. Cat# A14810 RRID: AB_2534323
PKP2- mutation analysis	Rabbit anti-PKP2	1:1000	Thermo Fisher Scientific Cat# PA5-53144, RRID:AB_2645606
GAPDH	Mouse anti-GAPDH	1:5000	Santa Cruz Biotechnology Cat# sc-32233, RRID:AB_627679
Differentiation Markers	Rabbit anti TBX6	1:50	Thermo Fisher Scientific Cat# PA5-35102, RRID:AB_2552412
	Rabbit anti NKX 2.5	1:100	Santa Cruz Biotechnology Cat# sc-14033, RRID:AB_650281
	Mouse anti Nestin	1:50	Millipore Cat# MAB5326, RRID:AB_2251134
	Rabbit anti SOX17	1:50	Thermo Fisher Scientific Cat# PA5-72815, RRID:AB_2718669
Secondary antibodies	Alexa Fluor 594 donkey anti rabbit IgG (H + L)	1:500	Molecular Probes Cat# A-21207, RRID:AB_141637
	Alexa Fluor 488 donkey anti mouse IgG (H + L)	1:500	Molecular Probes Cat# A-21202, RRID:AB_141607
	Alexa Fluor 594 donkey anti mouse IgG (H + L)	1:500	Molecular Probes Cat# A-21203, RRID:AB_141633
	Alexa Fluor 488 donkey anti rabbit IgG (H + L)	1:500	Molecular Probes Cat# A-21206, RRID:AB_2535792
	Goat Anti-Mouse HRP	1:15000	Agilent (Dako) Cat# P0447, RRID:AB_2617137
	Goat anti Rabbit HRP	1:10000	Thermo Fisher Scientific Cat# G-21234, RRID:AB_2536530
Nuclear stain	DAPI	300 nM	Thermo Fisher Scientific Cat# D1306, RRID:AB_2629482
Site-specific nuclease			
Nuclease information	Nuclease type/version	pSpCas9(BB)-2A-Puro (PX459) V2.0 (addgene: 62988)	
Delivery method	Lipofection	Lipofectamine Stem Transfection Reagent	
Selection/enrichment strategy	Selection cassette(s)	Puromycin	
Primers and Oligonucleotides used in this study			
	Target	**Forward/Reverse primer (5′–3′)**	
e.g. Pluripotency Markers (qPCR)	NANOG	AAAGGATCTTCACCTATGCC/GAAGGAAGAGGAGAGACAGT	
	OCT4	CTGGGTTGATCCTCGGACCT/CACAGAACTCATACGGCGGG	
e.g. House-Keeping Genes (qPCR)	RPL7	AATGGCGAGGATGGCAAG/TGACGAAGGCGAAGAAGC	
	GAPDH	ACAGCCTCAAGATCATCAG/GAGTCCTTCCACGATACC	
Genotyping	PKP2	GGATACCATGGAAAACTAGGGATGT/TGCTGTCAAAAACGGTGTCG	
Potential random integration-detecting PCRs	PKP2-sgRNA RV	TGCTGTCAAAAACGGTGTCG	
	U6 FW	ATAATTTCTTGGGTAGTTTGC	
gRNA oligonucleotide	PKP2-sgRNA	CCGTGGAAGAAAGGTCCTTG/CAAGGACCTTTCTTCCACGG	
Genomic target sequence(s)	Including PAM	CCGTGGAAGAAAGGTCCTTGAGG chr12:32,878,454–32,878,476	
Bioinformatic gRNA on- and -off-target binding prediction tool used	Benchling	https://benchling.com/s/seq-S3I7cT0h6FqR423aLTnC?m=slm-vl5CmksZqv7i4hyTgEdE	
Primers for top off-target mutagenesis predicted site sequencing	Off-Target1	TCTTTGCACTCATCCCCACC/AGCAGCCAGGATGTTTCTCC	
	Off-Target2	AGTTAGGAGGCAGGGCTTTG/CTAGGGTGCATGGAGGTTGG	
	Off-Target3	TGTCCTATCAGCACGACACA/ACTCTTATTCCCTTTCAGGTCCTT	
ODNs used as templates for HDR-mediated site-directed mutagenesis.	ssODN	ACAGTACAGCTCCCAGAAGTCCGTGGAAGAAAGGTTCTTGAGACATCCTCTGAGAAGACTGGAGATTTCT	

two PKP2-edited iPSCs lines, one with a point mutation (p.S140F) and another one with a premature stop codon using CRISPR/Cas9 gene editing technology.

In order to generate the PKP2 KO iPSC line, a gRNA to allow the Cas9 to target the PKP2 gene was used (PKP2-gRNA). For the PKP2 S140F edition a 70 nucleotide single strand DNA template (ssODN) complementary to the target sequence with the desired point mutation was co-transfected with the same gRNA (Fig. 1A).

After puromycin selection and clonal expansion of single cell derived colonies, genomic samples were taken for Sanger sequencing analysis. One iPSC clonal cell line carrying an homozygous mutation with the desired edition [PKP2: c.419C > T (p.S140F)] and another iPSC clonal cell line with the PKP2 KO as a result of an homozygous insertion of a one nucleotide [PKP2: c.422 + C (p.153X)], were selected (Fig. 1B). In the PKP2 KO cell line the codon frameshift generated a premature stop codon that caused the loss of the PKP2 protein, which was confirmed by western blot (Fig1H). Both clonal cell lines showed normal iPSCs morphology and no numerical or structural chromosomal alterations after karyotype analysis (Fig. 1C-D). Moreover, pluripotency was confirmed by OCT4 and SOX2 immunofluorescence staining (Fig. 1D), positive SSEA-4 expression evaluated by flow cytometry (Fig. 1E) and high expression of pluripotent genes OCT4, SOX2 and NANOG by RT-qPCR (Fig. 1F). In-vitro non-directed differentiation of both lines into cells from the three germinal layers was determined by immunofluorescence staining of NES (ectoderm), NKX 2.5 (mesoderm) and SOX17 (endoderm) (Fig. 1G). Short Tandem Repeat (STR) profiling confirmed the genetic identity of PKP2 S140F and PKP2 KO iPSCs with the wild type cells iPSC-FN2.1 (Questa et al., 2016). Finally, both iPSC clonal cell lines were negative for mycoplasma (See Table 1 and Table 2).

4. Materials and methods

4.1. iPSCs cell culture

iPSCs were maintained on Vitronectin (Gibco) coated wells with E8 Flex medium (Gibco). Every three days, cells were passed using PBS (Gibco), versene (Gibco) and media supplemented with 10uM Y27632 Rock inhibitor (Tocris). Cells were cultured in a 37 °C, 5% CO_2 and 90% humidity incubator.

4.2. CRISPR/Cas9

The PKP2-gRNA and the ssODN template for HDR were designed using Benchling software (https://www.benchling.com). Two complementary oligonucleotides codifying the PKP2-gRNA were synthesized, annealed and cloned into the plasmid pSpCas9(BB)-2A-Puro (PX459) V2.0. For plasmid and ssODN transfection, 100.000 iPSCs were plated on a geltrex-coated 12-multiwell dish. After 24 h, both DNA constructs were supplied to the cell culture using lipofectamine. The next day, medium was changed to mTeSR (StemCell) supplemented with 0,5 μg/ml Puromycin and Rock inhibitor for 48 h. Once the transfected cells were grown and expanded, clonal colonies were generated by single-cell selection cultured in a precoated well of a 96-well plate. iPSCs were collected for cryopreservation and DNA isolation.

4.3. Genotyping, sequencing and STR analysis

Genomic DNA was isolated from every iPSCs clonal cell line and the mutation site was amplified by PCR. PCR products were purified and sequenced in Macrogen. STR analysis of 27 locations was performed at the Laboratorio de Huellas Digitales Genéticas (Facultad de Farmacia y Bioquímica, UBA, Buenos Aires, Argentina).

4.4. Karyotyping

Chromosomal G-band analysis was performed by Laboratorio de Genética Hematológica, Instituto de Medicina Experimental (IMEX-CONICET)/Academia Nacional de Medicina. 50 metaphases were analyzed at 450-band resolution.

4.5. Western blot

Cell pellets were lysed with RIPA buffer. The proteins (20 μg) were loaded on a 10% polyacrylamide gel. They were then transferred on a nitrocellulose membrane and stained with PKP2 and GAPDH antibodies. Chemiluminescence was visualized using SuperSignal West Femto (Thermo Fisher), the software used was Image Quant 5000.

4.6. Immunofluorescence

Cells were fixed with 4% paraformaldehyde (Sigma). They were permeabilized and blocked with PBS-0.1% Triton X-100 and 3% normal goat serum, and incubated with primary antibodies overnight at 4 °C. Secondary antibodies were incubated for 1 h at RT and counterstained with DAPI. Cells were examined under an Evos XL Core inverted microscope.

4.7. Flow cytometry

iPSCs were dissociated into single cells and stained with SSEA-4 conjugated antibody for 1 h in the dark at RT.

BD Accuri C6 cytometer was used. Flow cytometry data were quantified using FlowJoX.

4.8. RNA isolation and RT-qPCR

cDNA was synthesized from 500 ng of RNA extracted with Trizol using MMLV reverse transcriptase (Promega). qPCR analysis was performed with StepOnePlus Real Time PCR System (PE Applied Biosystems). The FastStart Universal SYBR Green Master Mix (Roche) was used for all the reactions. Then mRNA values were analyzed using LinRegPCR and normalized against two housekeeping (RPL7 and GAPDH).

4.9. Non-direct differentiation

Two thousand cells were seeded in a single cell suspension in a non-coated dish in DMEM-20 %FBS (Gibco) supplemented with 50 μg/ml PenStrep (Gibco) and Rock inhibitor. After 48hs cells were transferred into 0.1% gelatin coated 24-well plate and cultured for 14 days.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2023.103157.

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