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Lab resource: Stem Cell Line

Generation of a gene-corrected human isogenic line (UAMi006-A) from propionic acidemia patient iPSC with an homozygous mutation in the *PCCB* gene using CRISPR/Cas9 technology

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ABSTRACT

Propionic acidemia (PA) is an inherited metabolic disease caused by mutations in the *PCCA* and *PCCB* genes. We have previously generated an induced pluripotent stem cell (iPSC) line (UAMi004-A) from a PA patient with the c.1218_1231del14ins12 (p.Gly407Argfs*14) homozygous mutation in the *PCCB* gene. Here, we report the generation of the isogenic control in which the mutation was genetically corrected using CRISPR/Cas9 technology. Off-target editing presence was excluded and the iPSCs had typical embryonic stem cell-like morphology and normal karyotype that expressed pluripotency markers and maintained their *in vitro* differentiation potential.

1. Resource Table

Unique stem cell lines identifier	UAMi006-A
Alternative name of stem cell line	PCCB10-FiPS4F-1-genetically corrected
Institution	Centro de Biología Molecular Severo Ochoa UAM-CSIC, Universidad Autónoma de Madrid, CIBERER, IDIPaz, Madrid, Spain.
Contact information of distributor	Eva Richard, erichard@cbm.csic.es
Type of cell line	iPSC
Origin	Human
Additional origin info	Age: 24 Sex: Female Ethnicity if known: Caucasian
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogramming	Sendai Virus
Genetic modification	Yes
Type of modification	Gene correction (isogenic control)
Associated disease	Propionic acidemia
Gene/locus	*PCCB*/3q22
Method of modification	CRISPR/Cas9
Name of transgene or resistance	Non applicable
Inducible/constitutive system	Non applicable
Date archived/stock date	May 2020
Cell line repository/bank	Spanish National Bank of Cell Lines http://www.isciii.es/ISCIII/es/contenidos/fd-el-instituto/fd-organizacion/fd-estructura-directiva/fd-subdireccion-general-investigacion-terapia-celular-medicina-regenerativa/fd-centros-unidades/fd-banco-nacional-lineas-celulares/fd-lineas-celulares-disponibles/lineas-de-celulas-iPS.shtml
Ethical approval	Patient informed consent obtained. Ethics Review Board-competent authority approval obtained (CEI 71–1278)

2. Resource utility

Currently, the molecular mechanisms underlying PA progression are not yet fully understood and there is no effective treatment for this disease. This iPSC line represents an ideal control to eliminate genetic background effects that exist between individuals for further mechanistic study and therapy evaluation.

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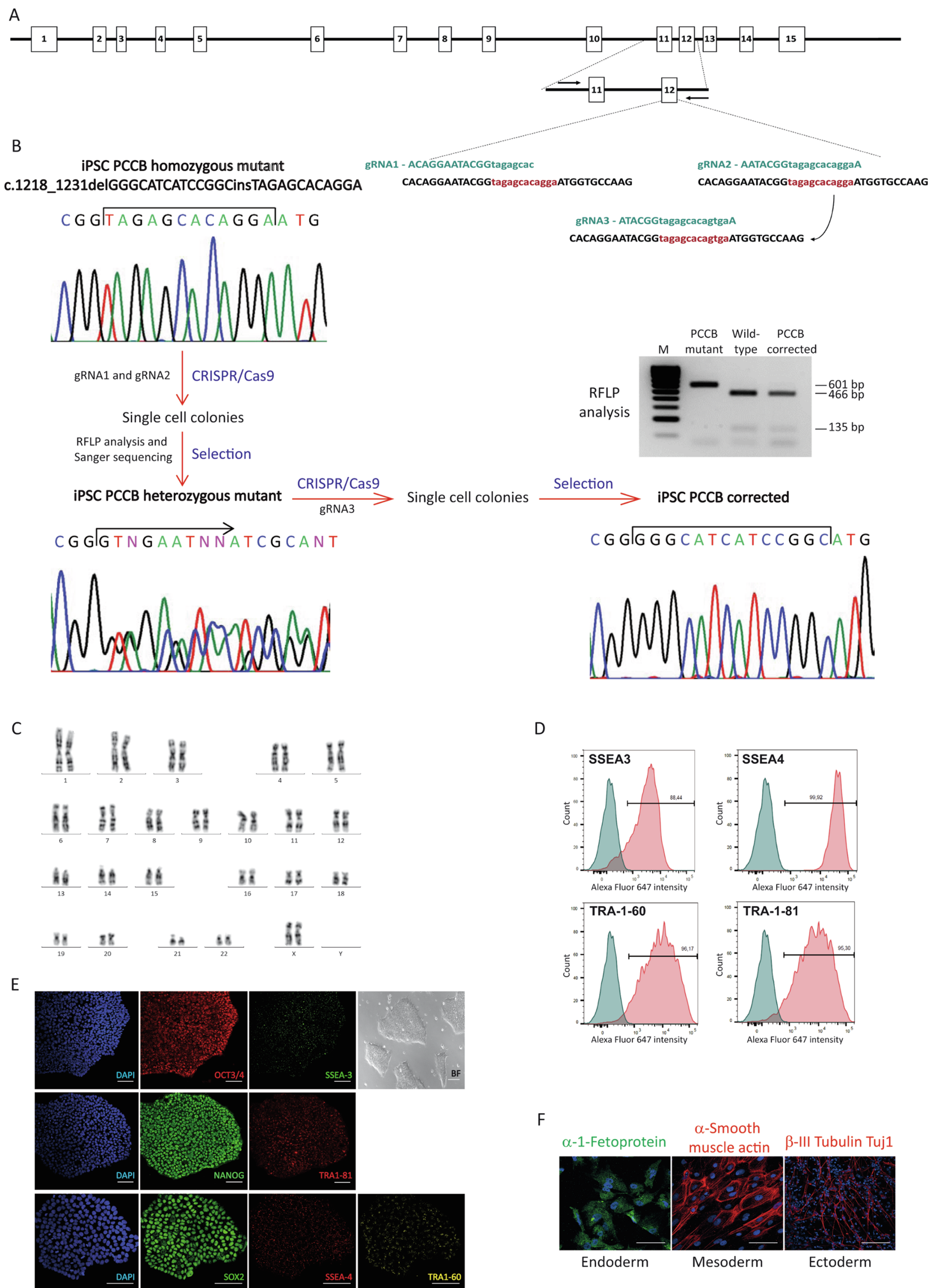

Fig. 1. Characterization of the iPSC line UAMi006-A. A) Specific RNA guides (gRNA 1, 2 and 3) designed in this study. B) Schematic demonstration of the mutation correction workflow. C) Karyotype analysis. D) Flow cytometry analysis for SSEA-3/SSEA-4/TRA-1-60 and TRA-1-81 pluripotency markers. E) Immunofluorescence analysis for OCT4/NANOG/SOX2/SSEA-3/SSEA-4/TRA-1-60 and TRA-1-81 pluripotency markers. F) *In vitro* differentiation analysis by immunofluorescence for α-1 fetoprotein (AFP), α-smooth muscle actin (SMA) and β-III tubulin (TUJ1) proteins.

3. Resource details

Propionic acidemia (PA, MIM#606054, ORPHA:35) is one of the most frequent life-threatening organic acidemias, caused by mutations in either the *PCCA* or *PCCB* genes, encoding both subunits of the mitochondrial propionyl-CoA carboxylase (PCC) enzyme, and is characterized by the toxic accumulation of propionyl-CoA and derived metabolites (Richard et al., 2015). Our group has previously generated two iPSC lines from PCCA (UAMi001-A) (Alonso-Barroso et al., 2017) and PCCB (UAMi004-A) (López-Márquez et al., 2019) patients to gain a deeper insight into PA pathophysiology and for evaluation of new therapeutic compounds. For that purpose, we aimed to generate the isogenic gene-corrected cell line by gene editing technology with CRISPR/Cas9, using a single-stranded donor template (ssDNA) to replace the PCCB homozygous variant, c.1218_1231del14ins12, with the wild-type sequence (Bollen et al., 2018). Specific RNA guides (gRNA 1, 2 and 3) were designed with the help of Breaking-Cas bioinformatic software based on location, editing score and off-targets percentage criteria (Fig. 1A). The off-targets sites presented in this work were selected according to score and genome localization criteria, taking into consideration only those located in intragenic regions. A ssDNA donor template carrying the wild-type sequence and 35 bp homology arms was used to drive the mutation correction. In a first approach, gene editing was performed using guides 1 and 2 and the results from restriction fragment length polymorphism (RFLP) assay and Sanger sequencing showed an heterozygous editing with guide 2 (Fig. 1B). So, a second round of editing was carried out using a third specific RNA guide (gRNA3). The final correction using gRNA3 was confirmed by RFLP and sequencing analysis (Fig. 1B) and no further mutation was detected in the 4 predicted off-target sites with highest scores after PCR followed by Sanger sequencing (data available with the authors). The gene-corrected UAMi006-A line presented normal hiPSC morphology (Fig. 1E, scale bar: 100 µm) and its karyotype integrity remained normal without detectable aberrations or rearrangements after the gene editing procedure (Fig. 1C). Pluripotency was evaluated by flow cytometry for SSEA-3/SSEA-4/TRA-1–60 and TRA-1–81 (Fig. 1D, Table 1) and by immunofluorescence for OCT4/NANOG/SOX2/SSEA-3/SSEA-4/TRA-1–60 and TRA-1–81 (Fig. 1E, scale bar: 88 µm) which was comparable to the parental iPSC line. Spontaneously differentiated cells were immunostained for endodermal market α-1 fetoprotein (AFP), mesodermal marker α-smooth muscle actin (SMA) and ectodermal marker β-III tubulin (TUJ1) confirming their *in vitro* differentiation capacity of forming all three germ layers (Fig. 1F, scale bar: 88 µm). The absence of mycoplasma contamination was verified by PCR (Supplementary Fig. S1). Furthermore, STR analysis of 16 loci showed 100% match between gene-corrected and parental iPSC lines (information available with the authors). In summary, an isogenic control (UAMi-006) from a PA iPSC line with PCCB defects (UAMi-004) has been successfully generated using CRISPR/Cas9 technology, meanwhile preserving its stem cell features (Fig. 1, Table 1).

4. Materials and methods

4.1. iPCS culture

iPSCs were cultured in mTESR™1 medium (StemCell™ Technologies) on plates coated with Matrigel® (Corning) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were passaged with ReleSR™ or with Accutase™ (both from StemCell™ Technologies) into a single cell suspension and resuspended in mTESR™1 with 10 µM Rock inhibitor (StemCell™ Technologies) every 3–4 days at 1:4 or 1:5 split ratio.

4.2. CRISPR/Cas9 gene correction

The web resource https://bioinfogp.cnb.csic.es/tools/breakingcas/ was used to design three guides with high specificity to *PCCB* target sites with the least number of predicted off-targets which were located in functional genome regions (Table 2). One day prior to nucleofection, cells were pretreated with mTSER™1 medium supplemented with CloneR 1X (StemCell™ Technologies). At day of nucleofection, 7×10^5 cells were nucleofected by Amaxa Nucleofector™ II Device, according to providers instructions, with ribonucleoprotein complexes formed by the combination of 11.5 µg of Cas9 (*Streptococcus pyogenes*, Integrated DNA Technologies (IDT)) and 3 µg of RNA duplex constituted by tracrRNA and RNA guides 1, 2 or 3 (IDT); and with 75 pmol of ssDNA (IDT) donor template (Table 2). Cells were transferred into one well of Matrigel-coated 24-well plate and cultured in 500 µl mTeSR™1 containing CloneR 1X. Medium was changed daily and CloneR was removed 24 h later. 3 days post-nucleofection, cells were dispersed at low density into Matrigel-coated 100 mm dishes in mTeSR™1 medium. About 7–10 days later, large colonies were picked and expanded.

4.3. Correction analysis

Genomic DNA was extracted from the iPSCs using QIAamp DNA

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography (Phase contrast microscopy)	Normal	Fig. 1 panel E
Phenotype	Qualitative analysis (Immunocytochemistry)	Positive for: OCT4, NANOG, SOX2, SSEA-3, TRA-1-81, SSEA-4 and TRA-1-60	Fig. 1 panel E
	Quantitative analysis (Flow cytometry)	SSEA-3: 88.5% SSEA-4: 99.9% TRA-1-60: 96.2% TRA-1-81: 95.3%	Fig. 1 panel D
Genotype	Karyotype (G-banding) and resolution	46XX Resolution 450-500	Fig. 1 panel C
Identity	STR analysis	16 sites tested and all of them matched	Available with the authors
Mutation analysis (IF APPLICABLE)	Sequencing	Homozygous c.1218_1231del14ins12 (p.Gly407Argfs*14) mutation in *PCCB* gene was corrected	Fig. 1 panel B
	Southern Blot OR WGS	Not performed	
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR: negative	Supplementary Fig. S1
Differentiation potential	Embryoid body formation	Expression of α-1-fetoprotein (endoderm), α-smooth muscle actin (mesoderm) and β-III-tubulin Tuj1 (ectoderm)	Fig. 1 panel F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	No
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	No
	HLA tissue typing	Not performed	No

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry

	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Mouse IgG anti-OCT4	1:60	Santa Cruz Cat# sc-5279, AB_628051
	Rat IgM anti-SSEA-3	1:3	Hybridoma Bank Cat# MC-631, AB_528476
	Rabbit IgG anti-SOX2	1:100	Fisher Thermo Scientific Cat# PA1-16968, AB_2195781
	Mouse IgG anti-SSEA-4	1:3	Hybridoma Bank Cat# MC-813-70, AB_528477
	Mouse IgM anti-TRA-1-60	1:200	Millipore Cat# MAB4360, AB_2119183
	Goat IgG anti-NANOG human	1:25	R&D Cat# AF1997, AB_355097
	Mouse IgM anti-TRA-1-81	1:200	Millipore Cat# MAB4381, AB_177638
Differentiation Markers	Rabbit IgG anti-α-Fetoprotein	1:400	Dako Cat# A0008, AB_2650473
	Mouse IgG anti-α-smooth muscle actin	1:400	Sigma-Aldrich Cat# A5228, AB_262054
	Mouse IgG anti-β-III-Tubulin Tuj1	1:500	Covance Cat# MMS-435P, AB_231377
Secondary antibodies	Alexa 555 Donkey anti-Mouse IgG	1:200	Thermo Fischer Cat# A-31570, AB_2536180
	Alexa 488 Goat anti-Rat IgM	1:200	Thermo Fischer Cat#A-21212, AB_2535798
	Alexa 488 Donkey anti-Rabbit IgG	1:200	Thermo Fischer Cat# A-31572, AB_162543
	Alexa 555 Donkey anti-Mouse IgG	1:200	Thermo Fischer Cat# A-31570, AB_2536180
	Alexa 647 Goat anti-Mouse IgM	1:200	Thermo Fischer Cat# A-21238, AB_2535807
	Alexa 647 Donkey anti-Goat IgG	1:200	Thermo Fischer Cat# A-21447, AB_2535864
	Cy3 Donkey anti-Mouse IgM	1:200	Jackson Cat# 715-165-140, AB_2340812
	Alexa 647 Goat anti-mouse IgG	1:600	Thermo Fischer Cat# A- 21235, AB_2535804

Primers

	Target	Forward/Reverse primer (5′-3′)
PCR and Sequencing	*PCCB* intron 10- intron 12 (exons 11 and 12: 601 bp)	GGATGGCTGCTGAGGACAAA/ TCCCACCACGGCTATGCTGTAG
Target sequences (gRNA)	*PCCB* exon 12 (c.1218_1231del GGGCATCATCCGGCins TAGAGCACAGGA)	gRNA1: ACAGGAATACGGTAGAGCAC gRNA2: AATACGGTAGAGCACAGGAA gRNA3: ATACGGTAGAGCACAGTGAA
ssDNA donor template	*PCCB* exon 12 (c.1218_1231del GGGCATCATCCGGCins TAGAGCACAGGA)	TCTTCCTCATGTCTAGGCACAGCACAGGAATACGG**GGGCATCATCCGGC**ATGGTGCCAAGCTTCTCTACGCATTTGCTGAGGCA
Off-targets PCR and sequencing analysis	AC090809.1 (gRNA2)	CTTCTAGATGACCTTGGCTCC/ GCATCTGAGGCTGCTTCTCTGT
		TGCCACCGAATGCTGCACAC/ CGAGGACATTGTAAGAGGTTGCC
	C16orf95 (gRNA3)	
		CAGACGATGCTGTTGCGGATG/ GGTTCCACCATGTTAGCCAG
	GALNT17 (gRNA3)	GGTCTGGCCAGTGAATAGG/ ACGGCTCTGAAGTTCACCAC
	LRP5 (gRNA3)	
Mycoplasma detection (PCR)	Mycoplasma species (986 bp: internal control band; and 520 bp: mycoplasma specific band)	Forward primers: CGCCTGAGTACGTTCGC CGCCTGAGTACGTACGC TGCCTGGGTAGTACATTCGC TGCCTGAGTAGTACATTCGC CGCCTGAGTAGTATGCTCGC CACCTGAGTAGTATGCTCGC CGCCTGGGTAGTACATTCGC Reverse primers: GCGGTGTGTACAAGACCCGA GCGGTGTGTACAAAACCCGA GCGGTGTGTACAAACCCCGA

Mini Kit (Qiagen) followed by PCR amplification using Supreme NZYTaq II 2x Green Master Mix kit (NZY) and the specific oligonucleotides indicated in Table 2. PCR was performed on a Veriti® Thermal Cycler (Applied Biosystems) using the following conditions: 95 °C, 5 min; 30 cycles of [95 °C, 30 s; 60 °C, 30 s; 72 °C, 15 s]; 72 °C, 7 min. PCR products were digested by MspI, and the fragments analyzed by agarose gels with ethidium bromide. Then, in positive clones, the *PCCB* gene fragment containing exons 11 and 12 was amplified and cloned in *pGEM®-T Easy Vector System I*. Clones (first round of editing) and PCR products (second round of editing) were sequenced by Sanger sequencing (Macrogen) to confirm the correction of the homozygous mutation in the *PCCB* gene.

4.4. Pluripotent markers and differentiation assays

iPSC were seeded onto matrigel-coated 15 μ-Slide 8 well culture plates (Ibidi), fixed with Formaline Solution 10% (Sigma-Aldrich), and stained with anti-OCT4/NANOG/SOX2/SSEA-3/SSEA-4/TRA-1-60 and TRA-1-81 as previously described (Alonso-Barroso et al., 2017). Images were obtained using a Zeiss confocal microscope. Pluripotency-associated markers SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 were also analyzed by flow cytometry as described (Alonso-Barroso et al., 2017) in a BD FACSCanto™ A instrument (Becton Dickinson) using FACSDiva 8.0 software. Unstained iPSCs and the corresponding isotype antibodies were used as negative controls to exclude data from non-specific

fluorescence. Embryoid body (EB) formation was applied by plating the dissociated cells onto 96-well v-bottom, low attachment plates (Deltalab). Emerging EB were replated on matrigel-coated 15 μ-Slide 8 well culture plates for another 15 days, fixed with Formaline Solution 10%, and stained with endodermal (AFP), mesodermal (SMA) and ectodermal (TUJ1) differentiation markers (Table 2).

4.5. *Karyotyping*

Cells were treated with 10 μg/ml Colcemid® Solution (Irvine Scientific) for 90 min at 37 °C, dissociated by accutase, treated with hypotonic solution, and fixed with Carnoy's fixative for karyotype analysis. At least 20 metaphases were karyotyped.

4.6. *STR analysis*

DNA fingerprinting analysis was performed as previously described (Alonso-Barroso et al., 2017) at Parque Científico de Madrid, Campus Moncloa, UCM, Madrid, Spain.

4.7. *Mycoplasma detection*

Mycoplasma test was performed using PCR method (Uphoff and Drexler, 2014). A positive sample with mycoplasma was used as a control. In Supplementary Fig. S1: positive control (C+).

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.

Acknowledgments

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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.2020.102055.

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