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Generation of isogenic Propionyl-CoA carboxylase beta subunit (PCCB) deficient induced pluripotent stem cell lines

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Abstract

Propionic acidemia (PA) is an autosomal recessive metabolic liver disease caused by defects in propionyl CoA carboxylase. Propionyl CoA carboxylase is a dodecameric enzyme consisting of multiple copies of alpha and beta subunits encoded by the PCCA and PCCB genes. Mutations in either PCCA or PCCB can cause the disease. PA is categorized as a rare disease and accessing patients' cells to study the disease has been challenging. Here we describe the generation of two isogenic induced pluripotent stem cell (iPSC) lines in which exon 2 of the PCCB gene was mutated using CRISPR Cas9 gene editing. The $PCCB^{-/-}$ iPSCs express characteristic pluripotency proteins, are competent to differentiate into cell lineages from each of the three embryonic germ layers and display a normal karyotype.

Resource Table:

Unique stem cell lines identifier	MUSCSDi001-A-1
	MUSCSDi001-A-2
Alternative names of stem cell lines	PCCB $^{\Delta 14/\Delta 29}$ (MUSCSDi001-A-1)
	PCCB $^{\Delta 19/\Delta 481}$ (MUSCSDi001-A-2)
Institution	Medical University of South Carolina
Contact information of distributor	Stephen Duncan, Duncanst@musc.edu
Type of cell lines	iPSCs that have been gene edited using CRISPR/Cas9
Origin	Human
Cell Source	Original cell type induced: Human foreskin fibroblast.
Clonality	Clonal
Method of reprogramming	Transgene free plasmid transfection
Multiline rationale	Isogenic clones
Gene modification	YES
Type of modification	Indel mutation (deletion)
Associated disease	Autosomal recessive propionic acidemia (PA)

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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.2020.101953.

Gene/locus	Exon2 of PCCB 3q22.3
Method of modification	CRISPR Cas9
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	2016–2019
Cell line repository/bank	The lines had been registered with hpscreg.eu with these unique identifiers, MUSCSDi001-A-1, MUSCSDi001-A-2
Ethical approval	Medical University of South Carolina Stem Cell Research Oversight Committee (SCRO) protocol 8.

1. Resource utility

The differentiation of PCCB deficient iPSC lines (Table 1) toward affected cell types, such as hepatocytes, will provide a cell culture model to study the mechanisms underlying PA and a platform for the discovery of potential therapeutics.

2. Resource details

Propionic acidemia (PA, OMIM #606054) is a rare inborn metabolic disease caused by loss of function mutations in the PCCA or PCCB genes that encode the alpha and beta subunits of the propionyl CoA carboxylase enzyme. In the mitochondria, Propionyl CoA carboxylase is responsible for conversion of propionyl CoA to methylmalonyl CoA during branched chain amino acid metabolism. Loss of enzyme activity leads to accumulation of toxic products in the mitochondria. Intermediate metabolites including propionic acid, methylcitrate and propionyl carnitine can be detected in the urine and blood of afflicted patients (Chapman et al., 2016). Here, we describe the generation of iPSCs in which the PCCB gene was disrupted using CRISPR Cas9 mediated gene editing. The parental iPSCs (iPSC-K3) were derived previously from human foreskin fibroblasts (ATCC CRL2097) using reprogramming factors encoding plasmids (Si-Tayeb et al., 2010). Human iPSCs-derived hepatocyte like cells have been used previously to study inborn errors in hepatic metabolism and as a platform for drug discovery (Pournasr and Duncan, 2017). We propose that iPSCs deficient in propionyl CoA carboxylase, will therefore be useful for the study of PA (Table 2).

A guide RNA was designed to target PCCB exon 2 (TGTTGAGAGCGACATGTTTG) and was cloned into a plasmid containing Cas9 from S. pyogenes in-frame with a T2A-Puromycin selectable marker (pSpCAs9(BB)-2A-Puro V2.0, PX459) as described elsewhere (Ran et al., 2013). Parental K3 iPSCs (Si-Tayeb et al., 2010) were electroporated with pSpCas9(BB)-2A-Puro-sgPCCB, followed by 48 h of selection in Puro containing medium. After 7–10 days individual colonies were manually collected. Half of each colony was used for genomic DNA extraction while the remainder was plated on a matrigel-coated plate. INDELs were identified by electrophoretic separation of PCR amplicons on acrylamide gels (Fig. 1A). Two clones harbouring compound heterozygous deletions were selected for further analyses. Nucleotide sequencing of the of PCR products (Fig. 1B) using primers described in Table 3 revealed that the MUSCSDi001-

A-1 carried a 14 bp deletion (g.5578_5592del, p.S82Rfs*92) on one allele and a 29 bp deletion (g.5563_5592, p.S78Tfs*87) on the other allele. MUSCSDi001-A-2 carried a 19 bp deletion (g.5582_5601del, p.M84Vfs*142) on one allele and a 481 bp deletion (g.5586_6067del, p.V86Sfs*142) on the other allele. (Fig. 1C, D). All these frameshift mutations were predicted to lead to the loss of propionyl CoA carboxylase, which was subsequently demonstrated by western blot analysis in iPSCs (Fig. 1E). The PCCB knockout iPSCs had a characteristic pluripotent morphology with a small cytoplasmic morphology, epithelial characteristic, and growth in colonies. Both iPSC lines expressed the pluripotency proteins Pou5f1 (also called Oct4), Nanog, and Sox2 (Fig. 1F). FACS analysis of Pou5f1 showed comparable levels to the parental cell line (Fig. 1G). Quantification of SOX2 immunostaining also showed more than %95 positive cells in both cell lines (Supplementary Fig. 1). Moreover, the cells were able to spontaneously differentiate as embryoid bodies into derivatives of all three germ layers (Fig. 1H). G-band chromosome analyses revealed that MUSCSDi001-A-1 had a normal karyotype (Fig. 1I). However, in iPSC MUSCSDi001-A-2 11 of 20 cells contained an unbalanced structural aberration in the long (q) arm of chromosome 9 (image on left Fig. 1J). The additional genetic material that had translocated to chromosome 9q, could not be characterized by G-banding. The cells (9 of 20 cells; image on right Fig. 1J) also contain an interstitial duplication in the long (q) arm of chromosome 20. There is a known recurrent acquired duplication at this location in human pluripotent stem cell cultures. Finally, both iPSC lines were confirmed to originate from K3 iPSCs using Short Tandem Repeat (STR) analysis and were confirmed to be free mycoplasma.

In summary, we have generated two iPSCs with deletions in the PCCB gene using CRISPR Cas9-mediated gene editing. Both lines displayed characteristics of iPSCs including the capacity to differentiate into all three germ layers. Although one of the lines had a normal chromosomal content and will be useful to examine the mechanistic basis of PA, the other line had genetic abnormalities that, if used, must be considered when interpreting any results.

3. Materials and methods

3.1. Cell culture

The iPSCs were cultured on an eCadherin-coated plate in mTeSR1 medium (Nagaoka et al., 2010) containing 40 ng/ml zebrafish fibroblast growth factor (zbFGF) in a humidified chamber at 37 °C, 4% O2, and 5% CO2. Cells were split one to ten approximately 4–6 days after reaching optimal confluency using Versene solution (Termofisher/Gibco, #15040-066). Cell lines were confirmed free of mycoplasma contamination using the Mycoplasma Detection PCR ELISA Kit (Roche, Germany, #11663925910).

3.2. CRISPR Cas9-mediated PCCB mutation

For *PCCB* mutation, 10×10^7 iPSCs were electroporated with 60 μg pSpCas9(BB)-2A-puro-sgPCCB plasmid using a BTX electroporator. pSpCas9(BB)-2A-Puro (PX459) V2.0 was a gift from Feng Zhang (Addgene plasmid # 62,988; http://n2t.net/addgene:62988; RRID:Addgene_62988). Soon after electroporation, cells were plated on Matrigel-coated plates in mTeSR1 supplemented with 10 μM Y-27632 (StemRD, CA, #146986-50-7).

Twenty-four hours later, medium was supplemented with 1 μg/ml puromycin and cells cultured for 48 h. The medium was then replaced with mTeSR1 and culture continued until individual colonies could be collected. Colonies were treated with 10 μM Y-27632 for 2 h before individual clones were collected by scratching. Around half of each colony was expanded in a single well from a 24-well Matrigel coated plate. The remainder of the colony provided genomic DNA for PCR analyses.

3.3. Genotyping and sequencing

Genomic DNA was extracted from iPSC clones using QuickExtract™ DNA extraction solution (Epicenter, WI, #QE09050). The targeted region of *PCCB* gene was amplified using Herculase Fusion Polymerase (Agilent, CA, #600675) with the primers listed in Table 3. PCR amplicons were run on Novex 4%–20% TBE gels (ThermoFisher/Invitrogen, CA, #EC6225BOX) to identify INDELs (Fig. 1A,B). Deletions were confirmed within amplicons by DNA sequencing and loss of protein was confirmed by western blot. Nucleotide sequencing was performed by Retrogen Inc. The results were aligned using SnapGene and CLC sequence viewer software (Fig. 1C, D).

3.4. Western blot

Whole cell lysates were collected from the iPSCs using RIPA buffer with protease inhibitor cocktail (ThermoFisher Scientific, NY, #78443). 20-30ug total protein was separated by SDS-PAGE using Any kD Mini-protean TGX stain-freeTM precast gels (BioRad, CA,#4568123), and transferred to PVDF membrane using the Trans-Blot TurboTM Transfer System (BioRad, CA, #1704155). Membranes were incubated overnight with antibodies listed at Table 3 at 4 °C. HRP-conjugated secondary antibodies were used at a dilution of 1:5000. Protein levels were quantified using BioRad stain-free Imaging System using Image Lab software from BioRad. Antibodies used are listed in Table 3.

3.5. Immunocytochemistry for pluripotency and differentiation markers

Cultured cells were fixed with 4% PFA for 20 min and made permeable using 0.4% Triton X-100 in PBS for 20 min. Cells were treated with 3% BSA in PBS for 1 h at room temperature followed by overnight incubation with the primary antibody at 4 °C. Cells were rinsed with PBS 3 X 5 min and incubated with secondary antibody for 1 h at room temperature. Control and experimental wells were processed identically.

Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Images were taken using IX51 Olympus fluorescence microscope and merged and quantified using Image J software. Antibodies used are listed in Table 3.

3.6. Embryoid body (EB) formation

The iPSCs were detached from eCadherin matrix using Versene solution (Life technologies, #15040–066) and transferred into suspension culture plates in complete mTESRl medium containing 10 μM Y-27632. Forty-eight hours after plating, Y-27632 was removed and spheres were cultured for another 8 days as suspension cultures in DMEM/Ham's F12 containing 20% knock-out serum replacement, NEAA and Pen/Strep without bFGF.

Thereafter, EBs were then transferred onto a gelatin or matrigel-coated plate and maintained in the same medium for another 8 days.

4. Karyotyping and cell authentication

G-banded karyotyping and Short Tandem Repeat Analyses were performed by WiCell .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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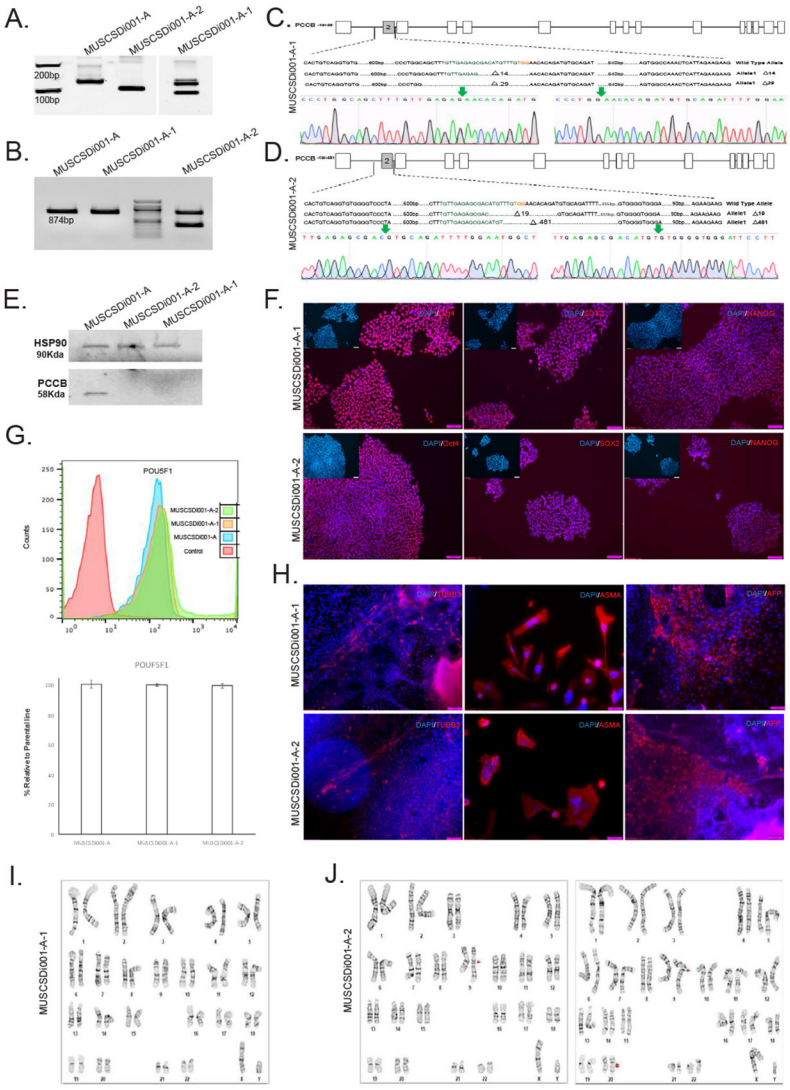

Fig 1.

Table 1

Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
MUSCSDi001-A-1	*PCCB* $^{\Delta 14/\Delta 29}$	*Male*	*Newborn*	*N/A*	Allele 1: g.5578_5592del Allele 2: g.5563_5592del	*Autosomal recessive propionic acidemia*
MUSCSDi001-A-2	*PCCB*$^{\Delta 19/\Delta 481}$	*Male*	*Newborn*	*N/A*	Allele 1: g.5582_5601del Allele 2: g.5586_6067del	*Autosomal recessive propionic acidemia*

Table 2

Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	*Normal*	*Data not shown*
Phenotype	Qualitative analysis by Immunocytochemistry	*Assess staining/expression of pluripotency markers: Pou5f1, Nanog, Sox2*	Fig. 1 *panel E*
	Quantitative analysis Flow cytometry	*Assess % of positive cells for cell surface marker Pou5f1 more that 85%*	Fig. 1 *panel F*
Genotype	Karyotype (G-banding) and resolution	MUSCSDi001-A-1: 46XY, Resolution 450–525 and MUSCSDi001-A-2: 46XY, add(9)(q21.2)[ll]/46,XY, add(9)(q21.2),dyp(20) (q11.2q11.2)[9]	Fig. 1 *panel H, I*
Identity	STR analysis	27 allelic polymorphisms across the 15 STR loci analyzed	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	*Compound heterozygous frameshift deletions in both cell lines*	Fig. 1 *panel A, B, and C.I*
Microbiology and virology	Mycoplasma	*Mycoplasma testing by PCR-ELISA. Negative*	Supplementary File-1
Differentiation potential	Embryoid body formation	*Mesoderm: smooth muscle actin*	Fig. 1 *panel GI*
		Ectoderm: β-III-tubulin	
		Endoderm α-fetoprotein	
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	*N/A*	*N/A*
Genotype additional info (OPTIONAL)	Blood group genotyping	*N/A*	*N/A*
	HLA tissue typing	*N/A*	*N/A*

Table 3

Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	*Rabbit anti-NANOG*	*1:200*	*Cell Signaling Technology Cat# 4903, RRID:AB_10559205*
	Rabbit anti-Sox2	*1:100*	*Abcam Cat# ab15830, RRID:AB_443255*
	Rabbit anti-Pou5f1	*1:100*	*Santa Cruz Biotechnology Cat# sc-9081, RRID:AB_2167703*
Differentiation Markers	*Mouse Anti-AFP*	*1:100*	*Sigma-Aldrich Cat# A8452, RRID:AB_258392*
	Rabbit Anti- αSMA	*1:100*	*Abcam Cat# ab124964, RRID:AB_11129103*
	Mouse Anti- TUBB3	*1:100*	*BioLegend Cat# 801202, RRID:AB_10063408*
Secondary antibodies	*Alexa flour 594 donkey anti rabbit*	*1:1000*	*Thermo Fisher Scientific Cat# A-21207, RRID:AB_141637*
	Alexa flour 488 donkey anti rabbit	*1:1000*	*Thermo Fisher Scientific Cat# A-21206, RRID:AB_2535792*
	Alexa flour 594 goat anti mouse	*1:1000*	*Thermo Fisher Scientific Cat# A-21203, RRID:AB_2535789*
			Thermo Fisher Scientific Cat# A-21203, RRID:AB_2535789
Primers			
	Target	**Forward/Reverse primer (5′-3′)**	
Genotyping	*PCCB*	*Fwd-120:GGAAAGCTAACAGCCAG/Rev120:CTTATTCTTATCAGCAGC*	
		Fwd131:TTCCATTGTAGGGAAAGC/Rev131:CTTATTCTTATCAGCAGC	
Targeted mutation analysis/sequencing	*PCCB*	*Fwd-XbaI:TTTTCTAGAGGAAAGCTAACAGCCAG/Rev-EcoRI:AACGAATTCCTTATTCTTATCAGCAGC*	
		Fwd-XbaI:TTTTCTAGATTCCATTGTAGGGAAAGA//Rev-EcoRI:AACGAATTCCTTATTCTTATCAGCAGC	
		Fwd615: CTTCCACAGTTGGGTGGCT /Rev615: AAGGCAGGGTACGGTCGCT	
		Fwd1230: CACTGTCAGGTGTGGGGTCCCT/Rev1230: ATGAGTTTGGCCACTATGCCTCA	