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Lab Resource: Stem Cell Line

Generation and characterization of a human iPSC line from a patient with propionic acidemia due to defects in the *PCCA* gene

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ABSTRACT

Human induced pluripotent stem cell (iPSC) line was generated from fibroblasts of a patient with propionic acidemia carrying mutations in the *PCCA* gene: c.1899+4_1899+7delAGTA; p.(Cys616_Val633del) and c.1430−−?_1643+?del; p.(Gly477Glufs*9). Reprogramming factors *OCT3/4*, *SOX2*, *KLF4* and *c-MYC* were delivered using a non-integrative method based on the Sendai virus. Once established, iPSCs have shown full pluripotency, differentiation capacity and genetic stability.

Resource table

Unique stem cell line identifier	UAMi001-A
Alternative name of stem cell line	PCCA23-FiPS4F8
Institution	Centro de Biología Molecular Severo Ochoa UAM-CSIC, Universidad Autónoma de Madrid, CIBERER, IDIPaz, Madrid, Spain
Person who created resource	Esmeralda Alonso-Barroso, Eva Richard
Contact person and email	Eva Richard, erichard@cbm.csic.es
Date archived/stock date	September 17, 2016
Origin	Human skin cells
Type of resource	Biological reagent: induced pluripotent stem cells (iPSC); derived from a patient with propionic acidemia due to defects in the *PCCA* gene
Sub-type	Human iPSC line
Key transcription factors	*OCT3/4*, *SOX2*, *c-MYC*, *KLF4* (CytoTune®-iPS 2.0 Sendai Reprogramming Kit; Invitrogen by Life Technologies)
Authentication	Identity and purity of cell line confirmed (Fig. 1)
Link to related literature	Not available
Cell line repository/bank	Not applicable
Ethics	Patient informed consent obtained/Ethics Review Board-competent authority approval obtained

Resource utility

This iPSC line was generated to differentiate into cardiomyocytes and neurons and obtain new disease models. We will investigate the pathophysiology of propionic acidemia disease and evaluate the effects of therapeutic compounds such as antioxidants.

Resource details

Propionic acidemia (PA) is an inherited metabolic disease caused by mutations in either the *PCCA* or *PCCB* genes (Richard et al., 2015). Fibroblasts from a compound heterozygous PA patient carrying two mutations in the *PCCA* gene (c.1899+4_1899+7delAGTA; p.(Cys616_Val633del) and c.1430−−?_1643+?del; p.(Gly477Glufs*9)) (Desviat et al., 2009) were reprogrammed using the CytoTune™ iPS 2.0 Sendai Reprogramming kit delivering the four human reprogramming factors *OCT3/4*, *SOX2*, *c-MYC* and *KLF4* (Takahashi et al., 2007). The iPSC line PCCA23-FiPS4F8 (UAMi001-A) displayed a typical round shape ESC-like morphology and growth behaviour (Fig. 1A) and the colonies stained positive for alkaline phosphatase activity (Fig. 1B). The clearance of the vectors and the exogenous reprogramming factor genes was observed by RT-PCR after 8 culture passages (Fig. 1C). Mycoplasma testing by a colorimetry assay revealed a negative result (Supplementary Fig. S1A). To analyze the genetic stability, we confirmed the presence of the two mutations in the iPSC line by Sanger sequencing (c.1899+4_1899+7delAGTA; p.(Cys616_Val633del) and multiplex ligation probe amplification (MLPA) analysis (c.1430−?_1643+?del; p.(Gly477Glufs*9)) revealing exons 17–18 deletion (Fig. 1D); and we also confirmed by DNA fingerprinting analysis that the line was derived from

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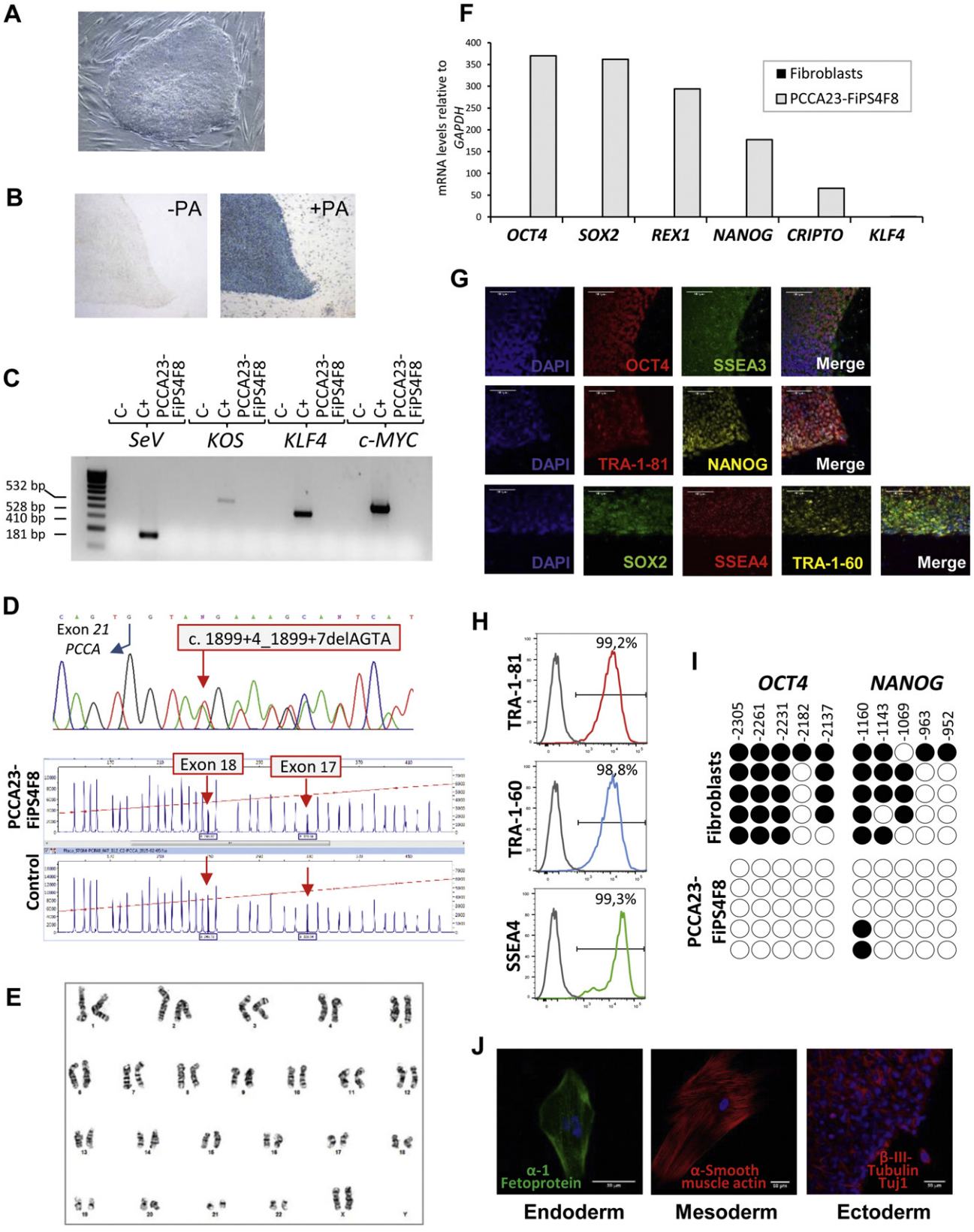
the patient's fibroblasts (Supplementary Fig. S1B). The iPSC line also displayed a normal karyotype (46, XX) after more than twenty culture passages (Fig. 1E). Expression of key pluripotency genes was observed both at RNA level (transcription factors *OCT4*, *SOX2*, *REX1*, *NANOG*, *CRIPTO* and *KLF4*) by qRT-PCR (Fig. 1F), as well as at protein level (transcription factors OCT4, NANOG and SOX2, and surface markers SSEA3, SSEA4, TRA-1-60 and TRA-1-81) by immunocytochemistry (Fig. 1G) and flow cytometry analysis (Fig. 1H). In addition, methylation analysis of the promoters of the



pluripotency associated genes, *OCT4* and *NANOG*, revealed a heavy methylation in the original fibroblasts and an almost complete demethylation in the iPSC line (Fig. 1I). Finally, the cells had the capacity to form derivatives of all three germ layers (endoderm, mesoderm and ectoderm) upon embryoid body differentiation (Fig. 1J, Table 1).

Materials and methods

Non-integrative reprogramming of mutant PCCA fibroblasts into iPSC

The present study included available fibroblasts from a PA patient with defects in the *PCCA* gene. Experimental protocols were approved by the Institutional Ethical Committee of the Universidad Autónoma de Madrid according to Spanish and European Union legislation, and informed consent was obtained from the legal care-givers. Patient-derived fibroblasts were cultured under standard conditions in MEM supplemented with 10% fetal bovine serum, 200 mM glutamine and antibiotics. Fibroblasts were reprogrammed using the CytoTune™ iPS 2.0 Sendai Reprogramming kit (Life Technologies) following the manufacturer's instructions. iPSCs were maintained and expanded both on feeder layers and on feeder-free layers. In the first case, human fibroblasts feeders were mitotically inactivated by gamma irradiation (80 Gy). iPSCs were cultured on top of irradiated human fibroblasts changing every day the iPSC medium containing: knockout DMEM (Life Technologies), 10% knockout serum replacement (Life Technologies), 0.5% human serum albumin (Grifols), 2 mM Glutamax ™ (Life Technologies), 100 μM non-essential amino acids (Lonza), 100 μM β-mercaptoethanol (Life Technologies), 50 U/ml penicillin, 50 mg/ml streptomycin (Life Technologies) and 10 ng/ml bFGF (Peprotech). For the propagation of iPSC, mechanical procedures were applied using a "stripper" micropipette and 150 μm tips (Mid Atlantic) to carefully lift and aspirate the colony. iPSC were also adapted and cultured in feeder-free conditions on Matrigel® (Corning) using iPSC conditioned medium and, in this case, for the iPSC propagation the enzymatic procedure StemPro® accutase® (Gibco by Life Technologies) was used. iPSC conditioned medium was obtained from feeder layers, supplemented with 10 ng/ml bFGF and filtered.

Phosphatase alkaline analysis

iPSC colonies were picked and plated onto a P-100 plate with feeders. After six days, direct phosphatase alkaline activity was determined using the Phosphatase Alkaline Blue Membrane Substrate Solution kit (Sigma-Aldrich) following manufacturer's instructions.

Detection of Sendai virus genome and transgenes

After 8 passages, iPSC line was tested for Sendai virus (SeV) residues. Total RNA was isolated using TRIzol® Reagent (Life Technologies) and 1 μg of RNA was retrotranscribed using NZY First-Strand cDNA Synthesis kit (NZY Tech). RNA from the transduced cell pool at passage zero was used as positive control. PCR was performed using the primers indicated in Table 2 and following the instructions as recommended by the manufacturer.

Mutation analysis

Genomic DNA from the patient-derived fibroblasts and iPSCs was isolated using MagNA Pure Compact DNA Isolation kit and MagNA Pure Compact instrument (Roche). Subsequently, amplification by PCR of the *PCCA* region containing the c.1899+4_1899+7delAGTA; p.(Cys616_Val633del) mutation was carried out using the primers indicated in Table 2, and amplified PCR fragments were sequenced in an ABI3730 sequencer (Applied Biosystems). Detection of c.1430−?_1643+?del; p.(Gly477Glufs*9) mutation was performed using the SALSA MLPA P278-C1-PCCA probemix (MRC-Holland) for copy number detection.

Karyotype analysis

Karyotype analysis of the iPSC line was carried out using cells with more than twenty culture passages which were processed using standard cytogenetic techniques. Briefly, cells were treated with 10 μg/ml of Colcemid® Solution (Irvine Scientific) for 90 min at 37 °C, dissociated by accutase, treated with hypotonic solution and fixed with Carnoy's fixative. Cells were then dropped on a microscope glass slide and dried. Metaphase cells were G banded using Wright staining. At least 20 metaphases were karyotyped. Karyotype analysis was performed at Instituto de Genética Médica y Molecular del Hospital Universitario de La Paz, Madrid, Spain.

Quantitative PCR analysis

Total RNA from the fibroblasts and iPSCs was isolated using TRIzol® Reagent (Life Technologies). RT-PCR was performed using High Capacity RNA to cDNA kit (Applied Biosystems), and real-time PCR was performed using SYBR® Green Master Mix (Applied Biosystems) and LightCycler 480 instrument (Roche), at Parque Científico de Madrid, Campus de Cantoblanco, UAM, Madrid, Spain. Amplification efficiency and sample-to-sample variation were normalized by monitoring *GAPDH*. The expression levels of several pluripotency associated genes (*OCT4*, *SOX2*, *REX1*, *NANOG*, *CRIPTO* and *HLF4*) were quantified. Primer sequences were described by (Aasen et al., 2008) (Table 2).

Immunofluorescence analysis

iPSC were grown on feeder layers on 15 μ-Slide 8 well culture plates (Aasen et al., 2008) and fixed with Formaline Solution 10% (Sigma-Aldrich). The following antibodies were used: TRA-1-60 (Millipore; MAB4360; 1:200); TRA-1-81 (Millipore; MAB4381; 1:200); SOX2 (Thermo Scientific, PA1-16968, 1:100); NANOG (RD Systems, AF1997, 1:25); SSEA-4 (DSHB, MC-813-70, 1:3); SSEA-3 (DSHB, MC-631, 1:3); OCT4 (Santa Cruz Biotechnology, sc-5279, 1:60); β-III Tubulin Tuj1 (Covance, MMS-435P, 1:500); α-1 Fetoprotein (Dako, A0008, 1:400); α-Smooth muscle actin (Sigma-Aldrich, A5228, 1:400) (Table 2). Secondary antibodies used were from Alexa Fluor Series from Jackson Immunoresearch, Thermo Fisher Scientific and Invitrogen (1:200) (Table 2). For nucleus staining DAPI (Invitrogen, 1:10,000) was used. Images were taken using a Zeiss confocal microscope.

Fig. 1. Generation and molecular and functional characterization of the PCCA23-FiPS4F8 iPSC line. (A) Typical embryonic stem cell-like colony morphology obtained after fibroblasts reprogramming. (B) Alkaline phosphatase enzymatic activity staining (right) and unstained iPS colony (left). (C) RT-PCR for the detection of the exogenous reprogramming factors and Sendai virus vectors. C+: transduced cell pool at passage zero; C−: non-template control. (D) Electropherogram showing mutation c.1899+4_1899+7delAGTA; p.(Cys616_Val633del) (top) and MLPA analysis showing the deletion of exons 17 and 18 (mutation c.1430−?_1643+?del; p.Gly477fs) (bottom) of the iPSC line. (E) Karyotype analysis. (F) qPCR showing the relative gene expression of the endogenous pluripotency associated markers *OCT4*, *SOX2*, *REX1*, *NANOG*, *CRIPTO* and *KLF4*. (G) Immunofluorescence analysis with typical embryonic stem cell markers such as transcription factors OCT4, NANOG and SOX2, and surface markers SSEA3, TRA-1-81, SSEA4 and TRA-1-60; scale bars: 100 μm. (H) Expression analysis of pluripotent markers (SSEA4, TRA-1-81 and TRA-1-60) by flow cytometry analysis. (I) Bisulfite sequencing analysis of *OCT4* and *NANOG* promoters. Each horizontal row of circles represents the methylation status of each CpG in one clone. Open circles indicate unmethylated CpG dinucleotides and filled circles, methylated. (J) Immunofluorescence analysis with specific markers of all three primary germ layers after *in vitro* differentiation. Endoderm: α-1-Fetoprotein; mesoderm: α-Smooth muscle actin; ectoderm: β-III-Tubulin Tuj1; scale bars: 50 μm.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Visual record of the line: normal	Fig. 1 panel A
Phenotype	Immunocytochemistry	Assess staining/expression of pluripotency and cell surface markers: OCT4, NANOG, SOX2, SSEA3, TRA-1-81, SSEA4 and TRA-1-60	Fig. 1 panel G
	Flow cytometry	Assess staining/expression of pluripotency markers: SSEA4, TRA-1-60, TRA-1-81	Fig. 1 panel H
Genotype	Karyotype (G-banding) and resolution	46XX, Resolution 450–500	Fig. 1 panel E
Identity	Microsatellite PCR (mPCR)	Not performed	
	STR analysis	16 sites tested and all of them matched	Supplementary Fig. 1 panel B
Mutation analysis (IF APPLICABLE)	Sequencing and MLPA analysis	c.1899+4_1899+7del AGTA; p.(Cys616_Val633del) and c.1430−?_1643+?del; p.(Gly477Glufs*9)	Fig. 1 panel D
	Southern Blot OR WGS	Not performed	
Microbiology and virology	Mycoplasma	Mycoplasma testing by a colorimetric assay: negative	Supplementary Fig. 1 panel A
Differentiation potential	Embryoid body formation	Expression of smooth muscle actin, β-III-tubulin Tuj1 and α − 1 fetoprotein	Fig. 1 panel J
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	
	HLA tissue typing	Not performed	

Flow cytometry analysis

We analysed the pluripotency-associated markers SSEA4, TRA-1-60 and TRA-1-81 by flow cytometry. iPSC were dissociated by incubation with accutase for 5 min. Then, cells were suspended in PBS/2%BSA to a cell density of 1.5×10^5 cells per 100 μl and incubated with the specific primary antibody (1/100 for TRA-1-60 and TRA-1-81, and no dilution for SSEA4) for 20 min at 4 °C. Cells were washed with PBS/2%BSA and incubated with Alexa Fluor® 647 (1/600) for 20 min at 4 °C. Finally, cells were washed with PBS/2%BSA and analysed using a FACSCanto A (Becton Dickinson) and FlowJo 10.2 software program. An irrelevant isotype-match antibody was always used as a negative control.

Bisulfite sequencing

Bisulfite modification of genomic DNA was performed with EZ DNA Methylation-Gold™ kit (Zymo Research) following the manufacturer's instructions. Converted DNA was amplified by PCR using primers previously published (Freberg et al., 2007) and Immolase™ Red DNA Polymerase (Bioline). PCR conditions were 95 °C for 8 min and 40 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, followed by 10 min at 72 °C. PCR products were cloned into bacteria by pGEM®-T Easy Vector (Promega) and sequenced using T7 primer. Sequences of 5 bacterial clones per genomic region examined are represented as rows of circles in Fig. 1H with each circle symbolizing the methylation state of one CpG.

Table 2
Reagents details.

Antibodies used for immunocytochemistry			
	Antibody	Dilution	Company Cat# and RRID
Pluripotency markers	Mouse IgG anti-OCT4	1:60	Santa Cruz Cat# sc-5279, AB_628051
	Rat IgM anti-SSEA3	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-SSEA4	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-β-III-Tubulin Tuj1	1:500	Covance Cat# MMS-435P, AB_231377
	Mouse IgG anti-α-smooth muscle actin	1:400	Sigma-Aldrich Cat# A5228, AB_262054
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
	Alexa 55 Donkey anti-Mouse IgM Cy3	1:200	Jackson Cat# 715-165-140,
Primers			
	Target	Forward/Reverse primer (5′-3′)	
Reverse transcription-PCR	*SeV* genome	GGATCACTAGGTGATATCGAGC/ACCAGACAAGAGTTTAAGAGATATGTATC	
	KOS transgene	ATGCACCGCTACGACGTGAGCGC/ACCTTGACAATCCTGATGTGG	
	KLF4 transgene	TTCCTGCATGCCAGAGGAGCCC/AATGTATCGAAGGTGCTCAA	
	c-MYC transgene	TAACTGACTAGCAGGCTTGTCG/TCCACATACAGTCCTGGATGATGATG	
Pluripotency Markers (Qpcr)	*OCT4*	GGAGGAAGCTGACAACAATGAAA/GGCCTGCACGAGGGTTT	
	SOX2	TGCGAGCGCTGCACAT/TCATGAGCGTCTTGGTTTTCC	
	NANOG	ACAACTGGCCGAAGAATAGCA/GGTTCCCAGTCGGGTTCAC	
	CRIPTO	CGGAACTGTGAGCACGATGT/GGGCAGCCAGGTGTCATG	
	REX1	CCTGCAGGCGGAAATAGAAC/GCACACATAGCCATCACATAAGG	
	KLF4	CGAACCCACACAGGTGAGAA/GAGCGGGCGAATTTCCAT	
House-keeping genes (qPCR)	*GAPDH*	GCACCGTCAAGGCTGAGAAC/AGGGATCTCGCTCCTGGAA	
Targeted mutation analysis/sequencing (PCR)	*PCCA*-exon 21	TTGATGGACATTTGGGTTTTT/AGCAGTAATGAAGCCAAGTTCA	

In vitro differentiation

To perform *in vitro* differentiation analysis, iPSC colonies were first cultured in suspension so that they form large aggregates called embryoid bodies (EBs). EBs differentiate spontaneously to different cell types derived from the three germ layers. iPSCs from a P100 plate treated with matrigel (80% confluency) were dissociated into a single cell suspension with accutase, and resuspended in 12 ml of conditioned medium. EBs formation was induced by seeding 120 μl of the iPSC suspension in each well of 96-well v-bottom low attachment plates, and by centrifuging the plates at 800*g* for 10 min to aggregate the cells. After 2 days, the EBs were transferred to untreated P60 culture plates for 2 days. Subsequently, the EBs were transferred to 15 μ-Slide 8 well culture plates previously treated with matrigel for 1 h at room temperature, and cultured in differentiation medium (DMEM supplemented with 20% fetal bovine serum, 2 mM Glutamax™, 100 μM non-essential amino acids, 100 μM β-mercaptoethanol, and 50 U/ml penicillin, 50 mg/ml streptomycin) for 2–3 weeks to allow spontaneous endoderm formation. For mesoderm differentiation, EBs were maintained for 2–3 weeks in differentiation medium supplemented with 100 μM ascorbic acid (A4403, Sigma-Aldrich). For ectoderm differentiation, EBs were cultured in a special differentiation medium containing (50% DMEM F12, 50% neurobasal medium, 2 mM Glutamax™, 1× N2 supplement, 1× B27 supplement and 50 U/ml penicillin, 50 mg/ml streptomycin, all from Gibco by Life Technologies) for 2–3 weeks. In all cases, the medium was changed every two days.

Mycoplasma detection

Cells were screened for mycoplasma contamination using a colorimetric assay, PlasmoTest™ Mycoplasma Detection Kit (InvivoGen), following the manufacturer's protocol.

DNA fingerprinting analysis

DNA fingerprinting analysis was performed using the AmpFLSTR® Identifiler® PCR Amplification Kit (Thermo Fisher Scientific). A total of 1 ng of DNA was used and highly polymorphic regions containing short tandem repeated sequences were evaluated by the amplification of the following markers (D8S1179, D21S11, D7S820, CSF1P0, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA and Amelogenin for sex determination) by PCR. Samples were run on a 3730 DNA Analyzer (Applied Biosystems) and the analysis was performed using GeneMapper® v4.0, at Parque Científico de Madrid, Campus Moncloa, UCM, Madrid, Spain.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2017.07.021.

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