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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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—7,1643+7del; p.(Gly477Glufs*9)) were reprogrammed using the CytoTune™ iPS 2.0 Sendai Reprogramming Kit delivering the four human reprogramming factors OCT3/4, SOX2, KLF4 and 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 c.1430—7,1643+7del; p.(Gly477Glufs*9)) revealing exons 17—18 deletion (Fig. 1D); and we also confirmed by DNA fingerprinting analysis that the line was derived from...
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. 1). Finally, the cells had the capacity to form derivatives of all three germ layers (endoderm, mesoderm and ectoderm) upon embryoid body differentiation (Fig. 1, 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 supplemented with 10% fetal bovine serum, 200 mM glutamine and informed consent was obtained from the legal care-givers. Patient-de

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→7.1463+?del; p.(Gly477Glufs*9) mutation was performed using the SALSA MLPA P278-1 -PCCA proxemix (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 (Irvin 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, RXF1, 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; 1:100); TRA-1-81 (Millipore; MAB4381; 1:200); SOX2 (Thermo Scientific, PAI-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); α-Fetoprotein (Dako, A0088, 1:400); α-Smooth muscle actin (Sigma-Aldrich, AS228, 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, 1:200) 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 iPSC 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→7.1463+7del; p.(Gly477Glufs*9) (bottom) of the iPSC line. (E) Karyotype analysis. (F) qPCR showing the relative gene expression of the endogenous pluripotency associated markers OCT4, SOX2, RXF1, NANOG, CRIPTO and HLF4. (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: α-III-Tubulin Tuj1; scale bars: 50 µm.
Flow cytometry analysis

We analysed the pluripotency-associated markers SSEA4, TRA-1-80 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 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 cells were washed with PBS/2%BSA and analysed using a FACSCanto A.

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 (Biolime). 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.
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 μl-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-Alrich). For ectoderm differentiation, EBs were cultured in a special differentiation medium containing (50% DMEM F12, 50% neurobasal medium, 2 mM Glutamax™, 1 x N2 supplement, 1 x 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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