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Lab Resource: Stem Cell Line

Generation of a human iPSC line from a patient with a defect of intergenomic communication

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ABSTRACT

Human iPSC line PG64SV.2 was generated from fibroblasts of a patient with a defect of intergenomic communication. This patient harbored a homozygous mutation (c.2243G>C; p.Trp748Ser) in the gene encoding the catalytic subunit of the mitochondrial DNA polymerase gamma gene (*POLG*). Reprogramming factors Oct3/4, Sox2, Klf4, and cMyc were delivered using a non integrative methodology that involves the use of Sendai virus.

1. Resource table

Name of stem cell line	PG64SV.2
Institution	Departamento de Bioquímica, Instituto de Investigaciones Biomédicas "Alberto Sols", Facultad de Medicina, (UAM-CSIC) and Centro de Investigación Biomédica en Red en Enfermedades Raras (CIBERER) Madrid, Spain. Instituto de Investigación Hospital 12 de Octubre ("i + 12"), Madrid, Spain.
Person who created resource	Francisco Zurita
Contact person and email	M. Esther Gallardo, egallardo@iib.uam.es
Date archived/stock date	July 20, 2014
Origin	Human skin cells
Type of resource	Biological reagent: induced pluripotent stem cells (iPSC) from a patient with a defect of intergenomic communication
Sub-type	Cell line
Key transcription factors	Oct3/4, Sox2, cMyc, Klf4
Authentication	Identity and purity of cell line confirmed (Fig. 1)
Link to related literature	None
Information in public databases	http://www.eurobiobank.org/en/services/services.htm

2. Resource details

The generation of the human iPSC line, PG64SV.2, was carried out using non-integrative Sendai viruses containing the reprogramming factors, Oct3/4, Sox2, cMyc, Klf4 (Takahashi et al., 2007). For this purpose, fibroblasts from a patient with a defect of intergenomic communication (Hirano et al., 2001) were provided by EuroBiobank. The patient's fibroblasts carried a homozygous mutation in the gene encoding the catalytic subunit of the mitochondrial DNA polymerase gamma (*POLG*) (c.2243G>C; p.Trp748Ser). The presence of this mutation in the iPSC line was confirmed by Sanger sequencing (Fig. 1A). PG64SV.2 iPSC colonies displayed a typical ES-like colony morphology and growth behavior (Fig. 1B) and they stained positive for alkaline phosphatase activity (Fig. 1C). We confirmed the clearance of the vectors and the exogenous reprogramming factor genes by RT-PCR after eight culture passages (Fig. 1D). The endogenous expression of the pluripotency associated transcription factors Oct4, Sox2, Klf4, Nanog, Crypto and Rex1 was also evaluated by RT-PCR (Fig. 1E). Immunofluorescence analysis revealed expression of transcription factors *OCT4*, *NANOG*, *SOX2* and surface markers *SSEA3*, *SSEA4*, *TRA1-60* and *TRA1-81* characteristics of pluripotent ES cells (Fig. 1F). Promoters of the pluripotency associated genes, *OCT4* and *NANOG*, heavily methylated in the original fibroblasts were almost demethylated in the PG64SV.2 line suggesting an epigenetic reprogramming to pluripotency (Fig. 1G). The iPSC line has been adapted to feeder-free culture conditions and displays a normal karyotype (46, XX) after more than twenty culture passages (Fig. 1H). We also confirmed by DNA fingerprinting analysis that the line PG64SV.2

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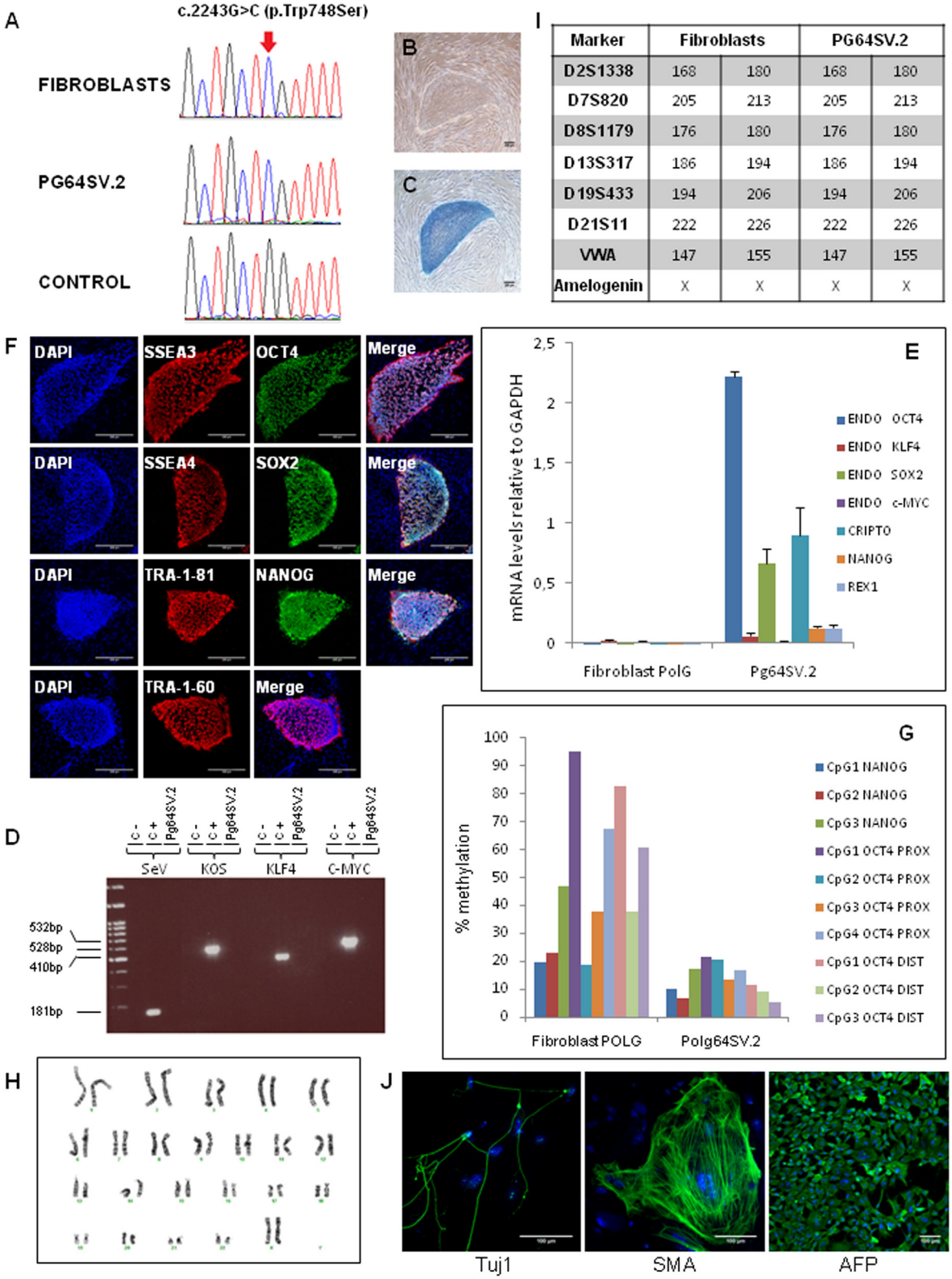

Marker	Fibroblasts		PG64SV.2	
D2S1338	168	180	168	180
D7S820	205	213	205	213
D8S1179	176	180	176	180
D13S317	186	194	186	194
D19S433	194	206	194	206
D21S11	222	226	222	226
VWA	147	155	147	155
Amelogenin	X	X	X	X

Fig. 1. Molecular and functional characterization of the PG64SV.2 iPSC line. 1A. Electropherograms showing the c.2243G > C mutation in the patient's fibroblasts and in the PG64SV.2 line. 1B. Typical ES-like colony morphology of the PG64SV.2 iPSC line. 1C. Positive phosphatase alkaline staining. 1D. Confirmation by RT-PCR of the exogenous reprogramming factors and sendai virus vector silencing. 1E. QPCR showing the expression of the pluripotency associated markers *NANOG*, *OCT4*, *SOX2*, *KLF4*, *CRIPTO* and *REX1*. 1F. Immunofluorescence analysis showing expression of typical pluripotent ES cell markers such as the transcription factors Oct4, Nanog, Sox2 and the surface markers SSEA3, SSEA4, Tra1-60 and Tra1-81; scale bars: 300 μm. 1G. Bisulfite pyrosequencing of the *OCT4* and *NANOG* promoters. The promoters of the transcription factors, OCT4 and NANOG were almost demethylated in the generated iPSC line. 1H. Karyotype analysis. PG64SV.2 has a normal karyotype (46, XX). 1I. DNA fingerprinting analysis showing that PG64SV.2 comes from the patient's fibroblasts. 1J. Embryoid body based *in vitro* differentiation assays. PG64SV.2 differentiates into all three germ layers, demonstrated by positive AFP endoderm staining (l), positive Tuj1 ectoderm staining and positive SMA mesoderm staining.

was derived from the patient's fibroblasts (Fig. 1I). Finally, the capacity of the generated iPSC line to differentiate into the three germ layers (endoderm, mesoderm and ectoderm) was tested *in vitro* using an embryoid body based assay (Fig. 1J).

3. Materials and methods

3.1. Non-integrative reprogramming of mutant POLG fibroblasts into iPSC

All the experimental protocols included in the present study were approved by the Institutional Ethical Committee of the Autonoma University of Madrid according to Spanish and European Union legislation.

Human fibroblasts from a patient presenting with a defect of intergenomic communication caused by a homozygous mutation in the *POLG* gene (c.2243G>C; p.Trp748Ser) were obtained from EuroBiobank (http://www.eurobiobank.org/en/services/services.htm). These fibroblasts were reprogrammed using the CytoTune-iPS 2.0 Sendai reprogramming kit following the instructions of the manufacturer. After eight passages of the iPSC line, silencing of the exogenous reprogramming factors genes and sendai virus genome was confirmed by RT-PCR following the manufacturer's instructions. PG64SV.2 was maintained and expanded both on feeder layers and on feeder-free layers. In the first case, irradiated human fibroblast feeders with ES medium containing: Knockout DMEM (Life technologies), Knockout serum replacement 20%, (Life technologies), MEM non-essential amino acids solution 1X (Life technologies), GlutaMAX 1X (Life technologies), β-mercaptoethanol (100 μM), penicillin/streptomycin 1X (Life technologies) and bFGF (4 ng/ml) (Miltenyi Biotec) were used. Subsequently, PG64SV.2 was adapted and cultured in feeder-free conditions on matrigel (354,277, Corning) with mTeSR1 medium (StemCell) following the recommendations of the manufacturer. For the propagation of the line, both enzymatic (dispase, collagenase IV and accumax) and mechanical procedures have been used.

3.2. Phosphatase alkaline analysis

The iPSC line PG64SV.2 was seeded on a feeder layer plate. After six days direct phosphatase alkaline activity was determined using the phosphatase alkaline blue membrane substrate solution kit (Sigma, AB0300) following the instructions of the manufacturer.

3.3. Mutation analysis

Total DNA from the patient's fibroblasts and iPSCs was extracted using a standard phenol-chloroform protocol. Subsequently, amplification by PCR of the of *POLG* gene region containing the c.2243G>C mutation (exon 13) was carried out using the following primers: POLG-13F: 5′- ATGGCCCTTGCTGAATGCAG-3′ and POLG-13R: 5′-TGGGCCTTGAGC AGAATGAG-3′. Following PCR amplification, direct sequencing of amplicons was performed on both strands in an ABI 3730 sequencer (Applied Biosystems; Foster City, CA) using a dye terminator cycle sequencing kit (Applera, Rockville, MD).

3.4. QPCR analyses

Total mRNA was isolated using TRIZOL and 1 μg was used to synthesize cDNA using the Quantitect reverse transcription cDNA synthesis kit. One microliter of the reaction was used to quantify by qPCR the expression of the endogenous pluripotency associated genes (*OCT4*, *SOX2*, *KLF4*, *NANOG*, *CRIPTO* and *REX1*). Primer sequences were described by Aasen et al., 2008. All the expression values were normalized to the *GAPDH* housekeeping gene. Plots are representative of at least three independent experiments.

3.5. Bisulfite pyrosequencing

Bisulfite modification of genomic DNA was performed with the EZ DNA Methylation-Gold kit (Zymo Research) following the manufacturer's instructions. The set of primers for PCR amplification and sequencing of *NANOG* and *OCT4* were designed using the software PyroMark Assay Design (version 2.0.01.15; Qiagen): Forward-*NANOG* (5′-TAT TGG GAT TAT AGG GGT GGG TTA-3′), Reverse-*NANOG* (5′-[Btn]- CCC AAC AAC AAA TAC TTC TAA ATT CAC-3′), and sequencing primer S-*NANOG* (5′-ATA GGG GTG GGT TAT-3′); Forward-*OCT4*_prox (5′- GGG GTT AGA GGT TAA GGT TAG TG-3′), Reverse-*OCT4*_prox (5′-[Btn]- ACC CCC CTA ACC CAT CAC-3′), and sequencing primer S-*OCT4*_prox (5′-GGG GTT GAG TAG TTT-3′); Forward-*OCT4*_dist (5′- TTT TTG TGG GGG ATT TGT ATT GA-3′), Reverse-*OCT4*_dist (5′-[Btn]- AAA CTA CTC AAC CCC TCT CT-3′), and sequencing primer S-*OCT4*_dist (5′-ATT TGT ATT GAG GTT TTG GA-3′). PCR was performed with primers biotinylated to convert the PCR product to single-stranded DNA templates, using the Vacuum Prep Tool. After PCR amplification, pyrosequencing reactions and methylation quantification were performed using PyroMark Q24 reagents, equipment and software (version 2.0.6; Qiagen), according to the manufacturer's instructions.

3.6. Karyotype analysis

Karyotype analyses of the iPSC line were carried out using cells with more than twenty culture passages. These cells were processed using standard cytogenetic techniques. Briefly, cells were treated with 10 μg/ml of Colcemid (Gibco) for 90 min at 37 °C, trypsinized, treated with 0.075 M hypotonic KCl 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.

3.7. Immunofluorescence analysis

Cells were grown on 0.1% gelatin-coated P35 culture plates (81,156, Ibidi) and fixed with 4% paraformaldehyde. The following antibodies for the staining were used: TRA-1-60 (Millipore; MAB4360; 1:150); TRA-1-81 (Millipore; MAB4381; 1:150); SOX2, (Thermo Scientific; PA116968; 1:100); NANOG (R&D Systems; AF1997; 1:25); SSEA-4 (Millipore; MAB4304; 1:10); SSEA-3 (Millipore; MAB4303; 1:10); OCT4 (Santa Cruz Biotechnology; Sc-5279; 1:100); neuron-specific class III beta-tubulin (Tuj1) (Sigma, T8660, 1:300), α-fetoprotein (AFP) (Sigma, WH0000174M1, 1:300), smooth muscle alpha actin (SMA) (Sigma, A2547, 1:400). Secondary antibodies used were all from the Alexa Fluor Series from Jackson Immunoresearch (all 1:500). Images were taken using a Zeiss confocal microscope.

3.8. In vitro differentiation assay

The *in vitro* pluripotency capacity of the iPSC line was tested by spontaneous embryoid body differentiation. For this purpose, iPSCs from a P100 plate with 80% of confluency were dissociated into a single cell suspension with accumax (SCR006,Millipore) and resuspended in 12 ml of mTeSR1 medium (Stemcell). Embryoid body formation was induced by seeding 120 μl of the iPSC suspension in each well of 96-well v-bottom low attachment plates and centrifuging the plates at 800 g for 10 min to aggregate the cells. After 2–3 days the embryoid bodies were transferred to an untreated P60 culture plate for 2–4 days. Subsequently, the embryoid bodies were transferred to 0.1% gelatin-coated P35 culture plates (81,156, Ibidi) and cultured in differentiation medium (DMEM F12 supplemented with 20% fetal bovine serum, 2 mM glutamine, 0.1 mM β-mercaptoethanol, 1X non essential amino acids and 1X penicillin–streptomycin, all from Invitrogen) for 2–3 weeks to allow spontaneous endoderm formation. For mesoderm differentiation, iPSCs were maintained for 2–3 weeks in differentiation

medium supplemented with 100 μM ascorbic acid (A4403, Sigma-Aldrich). For ectoderm differentiation, embryoid bodies were transferred to matrigel coated P35 culture plates and cultured in a special differentiation medium containing (50% DMEM F12, 50% neurobasal medium, 1X GlutaMAX, 1X penicillin–streptomycin, non-essential amino acids, 0.1 mM 2-mercaptoethanol, 1X N2 supplement and 1X B27 supplement, all from Invitrogen). In all the cases, the medium was changed every other day.

3.9. DNA fingerprinting analysis

For DNA fingerprinting analysis, highly polymorphic regions containing short tandem repeated sequences of DNA have been evaluated. For this purpose, the following markers (D13S317, D7S820, VWA, D8S1179, D21S11, D19S433, D2S1338 and amelogenin for sex determination) have been amplified by PCR and analyzed by ABI PRISM 3100 Genetic analyzer and Peak Scanner v3.5 (Applied Biosystems). Primer sequences and PCR conditions are available upon request.

Author disclosure statement

There are no competing financial interests in this study.

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