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Lab Resource: Stem Cell Line

Generation of a human iPSC line from a patient with Leigh syndrome caused by a mutation in the *MT-ATP6* gene

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ABSTRACT

Human iPSC line L749.1 was generated from fibroblasts of a patient with Leigh syndrome associated with a heteroplasmic mutation in the *MT-ATP6* gene. Reprogramming factors OCT4, SOX2, CMYC and KLF4 were delivered using retroviruses.

1. Resource table

Name of stem cell line	L749.1
Institution	Departamento de Bioquímica, Instituto de Investigaciones Biomédicas "Alberto Sols" (UAM-CSIC), Facultad de Medicina, Universidad Autónoma de Madrid 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	Teresa Galera-Monge
Contact person and email	M. Esther Gallardo, egallardo@iib.uam.es
Date archived/stock date	April 2013
Origin	Human skin cells
Type of resource	Biological reagent: Induced pluripotent stem cells (iPSC) from a patient with Leigh syndrome due to a mutation in the *MT-ATP6* gene
Sub-type	Cell line
Key transcription factors	OCT4, SOX2, CMYC, KLF4
Authentication	Identity and purity of cell line confirmed (Fig 1)
Link to related literature	None
Information in public databases	None

2. Resource details

The generation of the human iPSC line, L749.1, was carried out using retroviruses harboring the reprogramming factors, *OCT4*, *SOX2*, *CMYC*, *KLF4* (Takahashi et al., 2007). For this purpose, fibroblasts from a described patient presenting with Leigh syndrome and hypertrophic cardiomyopathy were obtained. The patient's fibroblasts carried a heteroplasmic mutation (90%) in the *MT-ATP6* gene (c.8993T>G; p. Leu156Arg) (Pastores et al., 1994). The presence of this mutation in the iPSC line was evaluated and confirmed by Sanger sequencing (Fig. 1A). L749.1 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 silencing of the retroviral transgenes by quantitative RT-PCR using primers specific for either the endogenous or transgenic factors *OCT4*, *SOX2* and *KLF4* (Fig. 1D). The pluripotency associated transcription factors *NANOG*, *CRIPTO* and *REX1* were also evaluated by RT-PCR (Fig. 1D). 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. 1E). Promoters of the pluripotency associated genes, *OCT4* and *NANOG*, heavily methylated in the original fibroblasts were almost demethylated in the L749.1 line suggesting an epigenetic reprogramming to pluripotency (Fig. 1F). The iPSC line has been adapted to feeder-free culture conditions and displays a normal karyotype (46, XY) after more than twenty culture passages (Fig. 1G). We also confirmed by DNA fingerprinting analysis that the line L749.1 was derived from the patient's fibroblasts (Fig. 1H). Finally, the capacity of the generated iPSC line to differentiate into the

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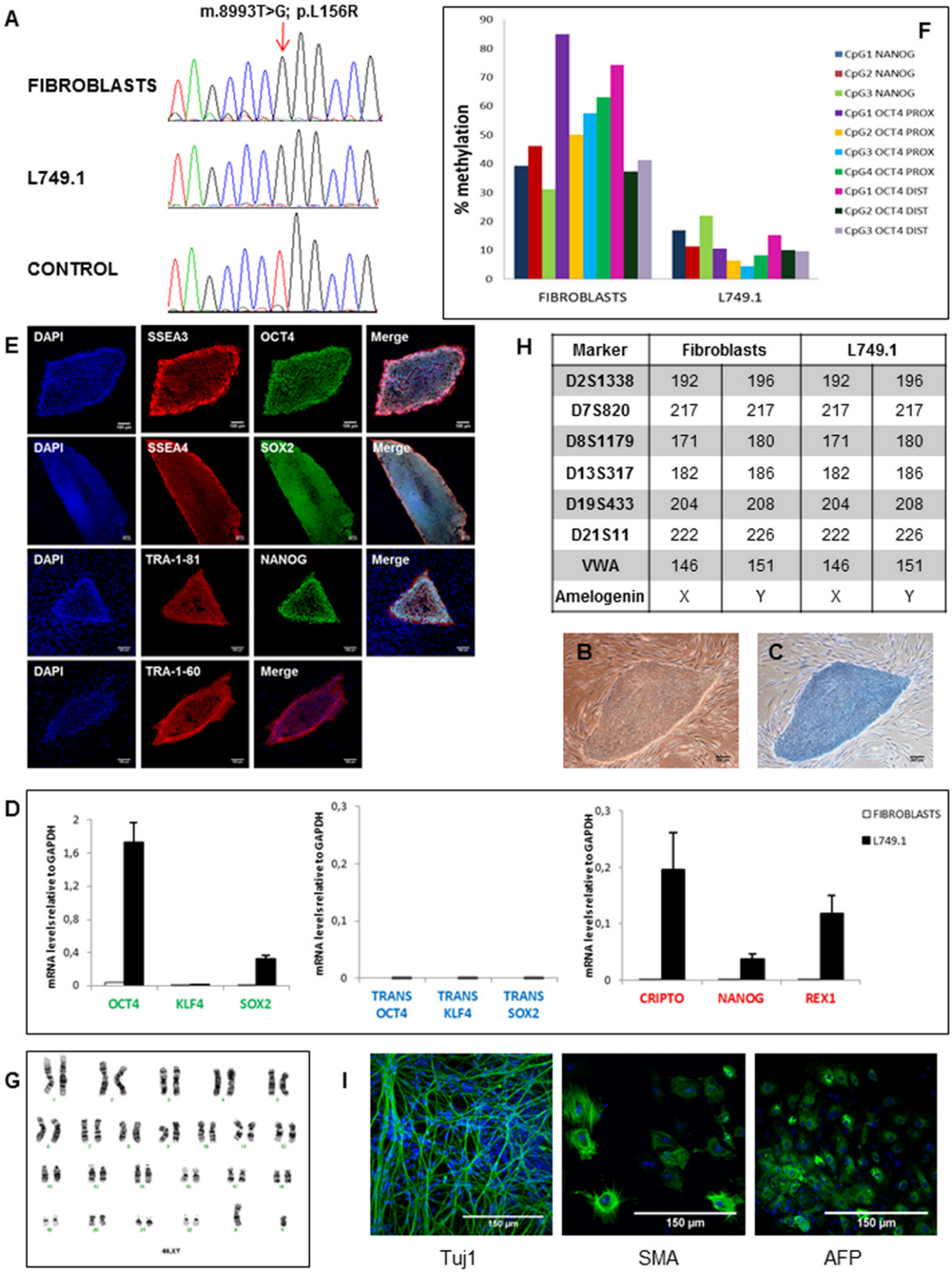

Marker	Fibroblasts		L749.1	
D2S1338	192	196	192	196
D7S820	217	217	217	217
D8S1179	171	180	171	180
D13S317	182	186	182	186
D19S433	204	208	204	208
D21S11	222	226	222	226
VWA	146	151	146	151
Amelogenin	X	Y	X	Y

Fig. 1. Molecular and functional characterization of the L749.1 iPSC line. A. Electropherograms showing the mutation in the patient's fibroblasts and in the L749.1 line. **B.** Typical ES-like colony morphology of the L749.1 iPSC line. **C.** Positive phosphatase alkaline staining. **D.** QPCR showing the expression of the pluripotency associated markers *NANOG*, *OCT4*, *SOX2*, *KLF4*, *CRIPTO* and *REX1*. **E.** 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: 300µm. **F.** 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. **G.** Karyotype analysis. L749.1 has a normal karyotype (46, XY). **H.** DNA fingerprinting analysis showing that L749.1 comes from the patient's fibroblasts. **I.** Embryoid body based *in vitro* differentiation assays. L749.1 differentiates into all three germ layers, demonstrated by positive AFP endoderm staining, positive Tuj1 ectoderm staining and positive SMA mesoderm staining.

three germ layers (endoderm, mesoderm and ectoderm) was tested *in vitro* using an embryoid body based assay (Fig. 1I).

3. Materials and methods

3.1. Reprogramming of mutant MT-ATP6 fibroblasts into iPSC using retroviruses

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 described patient presenting with Leigh syndrome and hypertrophic cardiomyopathy associated with a mutation in the *MT-ATP6* gene were acquired in Coriell (GM13411). Subsequently, these fibroblasts were reprogrammed into iPSCs. For this purpose pMSCV-based retroviral vectors expressing FLAG-tagged OCT4, SOX2, KLF4 and c-MYC, kindly provided by Dr. Raya, were used (Raya et al., 2010). The detailed protocol for the generation of iPSCs by retroviral transduction is described by Raya et al., 2010. After several passages of the iPSC line, silencing of the retroviral transgenes was confirmed by quantitative RT-PCR using primers specific for either the endogenous or transgenic factors. L749.1 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 1× (Life technologies), GlutaMAX 1× (Life technologies), β-mercaptoethanol (100 μM), penicillin/streptomycin 1× (Life technologies) and bFGF (4 ng/ml) (Miltenyi Biotec) were used. Subsequently, L749.1 was adapted and cultured in feeder-free conditions on matrigel (354277, 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 L749.1 was seeded on a feeder layer plate. After seven 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 *MT-ATP6* gene region containing the m.8993T>G mutation was carried out using the following primers: (MITO-13F: 5′-TTTC CCCCTCTATTGATCCC-3′ and MITO-13R: 5′-GTGGCCTTGGTATGTGCTTT-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 levels of transgenic and endogenous factors (*OCT4*, *SOX2* and *KLF4*) and the pluripotency markers (*NANOG*, *CRIPTO* and *REX1*). Primers 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 (81156, 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 (81156, Ibidi) and cultured in differentiation medium (DMEM F12 supplemented with 20% fetal bovine serum, 2 mM glutamine, 0.1 mM β-mercaptoethanol, 1× nonessential amino acids and 1× 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, 1× GlutaMAX, 1× penicillin–streptomycin, nonessential amino acids, 0.1 mM 2-mercaptoethanol, 1× N2 supplement and 1× 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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References

Aasen, T., Raya, A., Barrero, M.J., Garreta, E., Consiglio, A., Gonzalez, F., Vassena, R., Bilić, J., Pekarik, V., Tiscornia, G., Edel, M., Boué, S., Izpisua Belmonte, J.C., 2008. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat. Biotechnol. 26 (11), 1276–1284.

Pastores, G.M., Santorelli, F.M., Shanske, S., Gelb, B.D., Fyfe, B., Wolfe, D., Willner, J.P., 1994. Leigh syndrome and hypertrophic cardiomyopathy in an infant with a mitochondrial DNA point mutation (T8993G). Am. J. Med. Genet. 50 (3), 265–271.

Raya, A., Rodríguez-Pizà, I., Navarro, S., Richaud-Patin, Y., Guenechea, G., Sánchez-Danés, A., Consiglio, A., Bueren, J., Izpisua Belmonte, J.C., 2010. A protocol describing the genetic correction of somatic human cells and subsequent generation of iPS cells. Nat. Protoc. 5 (4), 647–660.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131 (5), 861–872.