Lab Resource: Stem Cell Line

Generation of a human iPS line from a patient with Leigh syndrome

Teresa Galera, Francisco Zurita, Cristina González-Páramos, Ana Moreno-Izquierdo, Mario F. Fraga, Agustin F. Fernández, Rafael Garces, M. Esther Gallardo

Abstract

Human iPS line LND554SV.3 was generated from heteroplasmic fibroblasts of a patient with Leigh syndrome carrying a mutation in the MT-ND5 gene (m.13513G > A; p.D393N). Reprogramming factors Oct3/4, Sox2, Klf4, and cMyc were delivered using a non-integrative methodology that involves the use of Sendai virus.

© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license.

Keywords:
Leigh syndrome
Induced pluripotent stem cells
iPS cells
MT-ND5

Resource table

Name of stem cell line: LND554SV.3
Institution: Departamento de Bioquímica, Instituto de Investigaciones Biomédicas “Alberto Sols”, Facultad de Medicina (UAM-CSIC), Spain and Centro de Investigación Biomédica en Red en Enfermedades Raras (CIBERER) Madrid, Spain
Person who created resource: Teresa Galera
Contact person and email: M. Esther Gallardo, egallardo@iib.uam.es
Date archived/stock date: June 20, 2013
Ethics: Patient informed consent obtained/Ethics Review Board-competent authority approval obtained

Resource details

The generation of the human iPS line, LND554SV.3, 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 described patient with Leigh syndrome, an inherited devastating neurodegenerative disorder, were employed (Monlleo-Neila et al., 2013). The patient’s fibroblasts carried a heteroplasmic mitochondrial DNA (mtDNA) mutation in the MT-ND5 gene (m.13513G > A; p.D393N) with a mutant mtDNA load of 55%. The presence of this mutation in the iPSCs was confirmed (Fig. 1A). Interestingly, the percentage of mutant mtDNA in the LND554SV.3 line was only 32% due to spontaneous segregation of the heteroplasmic mtDNA content (Fig. 1A). LND554SV.3 iPS 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, CRIPTO and REX1 was evaluated by quantitative...
A

**FIBROBLASTS**

m.13513G>A; p.D393N

**LND554SV.3**

**CONTROL**

B

I

<table>
<thead>
<tr>
<th>Marker</th>
<th>Fibroblasts</th>
<th>LND554SV.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2S1338</td>
<td>180</td>
<td>181</td>
</tr>
<tr>
<td>D7S920</td>
<td>209</td>
<td>220</td>
</tr>
<tr>
<td>D8S1179</td>
<td>167</td>
<td>172</td>
</tr>
<tr>
<td>D13S317</td>
<td>174</td>
<td>182</td>
</tr>
<tr>
<td>D19S433</td>
<td>194</td>
<td>198</td>
</tr>
<tr>
<td>D21S11</td>
<td>226</td>
<td>236</td>
</tr>
<tr>
<td>VWA</td>
<td>145</td>
<td>146</td>
</tr>
<tr>
<td>Amelogenin</td>
<td>X</td>
<td>Y</td>
</tr>
</tbody>
</table>

E

![Graph](image)

F

**DAPI**

**SSEA3**

**OCT4**

**Merge**

**DAPI**

**SSEA4**

**SOX2**

**Merge**

**DAPI**

**TRA-1-81**

**NANOG**

**Merge**

**DAPI**

**TRA-1-60**

**Merge**

D

**C**

**LND554SV.3**

SeV 181 bp 532 bp 410 bp 528 bp c-MYC KLF4 KOS

H

J

**Tuj1**

**SMA**

**AFP**
real time polymerase chain reaction (qPCR) (Fig. 1E). Immunofluorescence analysis revealed expression of transcription factors OCT4, NANO, 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 NANO, heavily methylated in the original fibroblasts were almost demethylated in the LND554SV.3 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, XY) after more than twenty culture passages (Fig. 1H). We also confirmed by DNA fingerprinting analysis that the line LND554SV.3 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).

Materials and methods

Non-integrative reprogramming of Leigh 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 described patient presenting with Leigh syndrome caused by a heteroplasmic mutation in the mtDNA (m.13513G→A; p.D393N) were kindly provided by Dr. Francina Munell from the Hospital Universitario Vall d’Hebron (Barcelona, Spain) (Monlelo-Neila et al., 2013). 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 factor genes and Sendai virus genome was confirmed by RT-PCR following the manufacturer’s instructions. LND554SV.3 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, LND554SV.3 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.

Phosphatase alkaline analysis

The iPSC line LND554SV.3 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.

Mutation analysis

Total DNA from the patient’s fibroblasts and iPSCs was extracted using a standard phenol-chloroform protocol. Subsequently, amplification by PCR of a mtDNA region containing the m.13513G→A mutation was carried out using the following primers: mt-20F: 5’ ATCTGTACCCACCT TC 3’ and mt-20R: 5’ AGAGGGGTCAAGGTTGATTCC 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).

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 μl of the reaction was used to quantify by qPCR the expression of the endogenous pluripotency associated genes (OCT4, SOX2, KLF4, NANO, 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.

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 NANO and OCT4 were designed using the software PyroMark Assay Design (version 2.0.01.15; Qiagen): Forward-NANO (5’-TAT TGG GAT TAT AGG GGT GGA TTA-3’), Reverse-NANO (5’-[Btm]-CCC AAC AAC TAC TCT TAA ATT CAC-3’), and sequencing primer S-NANO (5’-ATA ATG GTG TTG TAT TAT GAT TAT-3’); Forward-OCT4_prox (5’-GGG GTT AGA GGT TAA GGT TAG TG-3’), Reverse-OCT4_prox (5’-[Btm]-ACC CTA CCA ACC CAT CAC-3’), and sequencing primer S-OCT4_prox (5’-GGG GGT GAG TTA GAA TTT-3’); Forward-OCT4_dist (5’-TTT TGG TGG GAT TGT TGG TGT GGA-3’), Reverse-OCT4_dist (5’-[Btm]-AAA CTA CTC AAC CCC TCT CTT-3’), and sequencing primer S-OCT4_dist (5’-ATT TGT ATT GAG TTT TGT 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.

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 10μg/mL of Colcemid (Gibco) for 90 min at 37 °C, trypsinized, treated with hypotonic solution KCl 0.075 M, 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.

Immunofluorescence analysis

Cells were grown on 0.1% gelatin-coated P35 culture plates (81156, Ibidi) and fixed with 4% paraformaldehyde. The following antibodies for fluorescence analysis were used: TRA-1-60 (Millipore; MAB4360; 1:150); TRA-1-81 (Millipore; MAB4381; 1:150); SOX2, (Thermo Scientific; PA1-19698; 1:100); NANO (R&D Systems; AF1997; 1:25); SSEA-3 (Millipore; MAB4303; 1:10); OCT4 (Santa Cruz Biotechnologie; Sc-5279; 1:100); neuron-specific class III beta-tubulin (TUJ1) (Sigma, T8660; 1:300), α-fetoprotein (AFP) (Sigma, 85600; 1:500; Millipore), and cytokeratin (KLF4, CRIPTO and REX1) F. Immunofluorescence analysis showing expression of typical pluripotent ES cell markers such as the transcription factors OCT4, NANO, SOX2 and the surface markers SSEA3, SSEA4, TRA1-60 and TRA1-81; scale bars: 300 μm. G. Bisulfite pyrosequencing of the OCT4 and NANO promoters. The promoters of the transcription factors, OCT4 and NANO were almost demethylated in the generated iPSC line. H. Karyotype analysis. LND554SV.3 has a normal karyotype (46, XY). I. DNA fingerprinting analysis showing that LND554SV.3 comes from the patient’s fibroblasts. J. Embryoid body based in vitro differentiation assays. LND554SV.3 differentiates into all three germ layers, demonstrated by positive AFP endoderm staining (l), positive TUJ1 ectoderm staining and positive SMA mesoderm staining.

Fig. 1. Molecular and functional characterization of the LND554SV.3 iPSC line. A. Electropherograms showing the m.13513G→A mutation in the patient’s fibroblasts and in the LND554SV.3 line. B. Typical ES-like colony morphology of the LND554SV.3 iPSC line. C. Positive phosphatase alkaline staining. D. RT-PCR for detecting the cleavage of the vectors and the exogenous reprogramming factor genes. E. qPCR showing the expression of the pluripotency associated markers NANO, OCT4, SOX2, KLF4, CRIPTO and REX1. F. Immunofluorescence analysis showing expression of typical pluripotent ES cell markers such as the transcription factors OCT4, NANO, SOX2 and the surface markers SSEA3, SSEA4, TRA1-60 and TRA1-81; scale bars: 300 μm. G. Bisulfite pyrosequencing of the OCT4 and NANO promoters. The promoters of the transcription factors, OCT4 and NANO were almost demethylated in the generated iPSC line. H. Karyotype analysis. LND554SV.3 has a normal karyotype (46, XY). I. DNA fingerprinting analysis showing that LND554SV.3 comes from the patient’s fibroblasts. J. Embryoid body based in vitro differentiation assays. LND554SV.3 differentiates into all three germ layers, demonstrated by positive AFP endoderm staining (l), positive TUJ1 ectoderm staining and positive SMA mesoderm staining.
WH0000174M1, 1:300), smooth muscle alpha actin (SMA) (Sigma, A2547, 1:400). Secondary antibodies used were all from the Alexa Fluor Series (1:500) or from Jackson Immunoresearch (Cy2, 1:50; Cy3, 1:250). Images were taken using a Zeiss confocal microscope.

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, 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 aminoacids, 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.

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, DBS1179, 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.

Acknowledgments

We are grateful to Prof. Angel Raya for his help and advice with iPS cell generation. This work was supported by grants from the “Centro de Investigación Biomédica en Red en Enfermedades Raras” (CIBERER) (grant 13-717/132.05 to RG), the “Instituto de Salud Carlos III” [Fondo de Investigación Sanitaria and Regional Development Fund (ERDF/FEDER) funds PI10/0703 and PI13/00556 to RG and PI15/00484 to MEG], “Comunidad Autónoma de Madrid” (grant number S2010/BMD-2402 to RG); TG receives grant support from the Universidad Autónoma de Madrid (FPI-UAM) and FZD from the Ministerio de Educación, Cultura y Deporte (FPU13/00544). MEG is a staff scientist at the “Centro de Investigación Biomédica en Red en Enfermedades Raras” (CIBERER).

References

