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Lab Resource: Stem Cell Line

Generation of a human iPSC line from a patient with retinitis pigmentosa caused by mutation in *PRPF8* gene

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ABSTRACT

The human iPSC cell line, RP2-FiPS4F1 (RCPFi001-A), derived from dermal fibroblasts from the patient with retinitis pigmentosa caused by the mutation of the gene *PRPF8*, was generated by non-integrative reprogramming technology using OCT3/4, SOX2, CMYC and KLF4 reprogramming factors.

Resource table

Name of Stem Cell line	RP2-FiPS4F1(RCPFi001-A)
Institution	Research Center Principe Felipe, Eduardo Primo Yufera 3, Valencia, Spain
Person who created resource	Slaven Erceg
Contact person and email	Dr. Slaven Erceg, serceg@cipf.es
Date archived/stock date	July 14, 2016
Origin	Human dermal fibroblasts
Type of resource	Biological reagent: induced pluripotent stem cell (iPS)
Sub-type	Cell line
Key transcription factors	Oct3/4, Sox2, c-Myc, Klf4
Authentication	Identity and purity of cell line confirmed (Fig. 1)
Link to related literature	
Information in public databases	
Ethics	Patient informed consent obtained/Ethics Review Board-competent authority approval obtained

Resource details

Skin punch biopsy was taken from 42-year-old patient who was diagnosed with retinitis pigmentosa (RP) harboring c.6974_6994del21bp (p.V2325fsX2329) mutation in heterozygosis in pre-mRNA splicing factor 8 (*PRPF8*) (Martinez-Gimeno et al., 2003) and primary fibroblast cell line was established. The generation of the human induced pluripotent stem cell (hiPSC) line, RP2-FiPS4F1 (registered as RCPFi001-A at www.hPSCreg.com), was carried using non-integrative Sendai virus containing the human reprogramming factors, Oct3/4, Sox2, c-Myc, Klf4 following instructions by manufacturer. After 30 days generated colonies displayed a typical ES-like morphology (polygonal shape; refractive edges, high nuclear/cytoplasmic ratio) and growth behaviour. DNA sequencing analysis of RP2-FiPS4F1 confirmed the mutation in one allele in *PRPF8* (Fig. 1A). Pluripotency was additionally assessed by immunocytochemistry to pluripotency markers OCT-4, SOX2, NANOG and SSEA-4 (Fig. 1B). The alkaline phosphatase is known to be more active in hiPSCs and the colorimetric assay depicting its activity confirmed that the selected hiPSC colonies are indeed pluripotent. The clearance of the virus and the exogenous reprogramming factor genes was confirmed by RT-PCR after eight cell culture passages (Fig. 1C). The expression of endogenous pluripotency genes was detected by RT-PCR (Fig. 1D). The selected line was kariotypically normal at low and medium passages (Fig. 1E). The genetic fingerprinting was performed with RP2-FiPS4F1 hiPSC line and proved its genetic identity to parental fibroblasts. (Fig. 1F). To test the ability of generated hiPSC line to generate derivates of three germ layers *in vivo*, the RP2-FiPS4F1 cells were transplanted subcutaneously into the immunodeficient (SCID) mice. Eight weeks after

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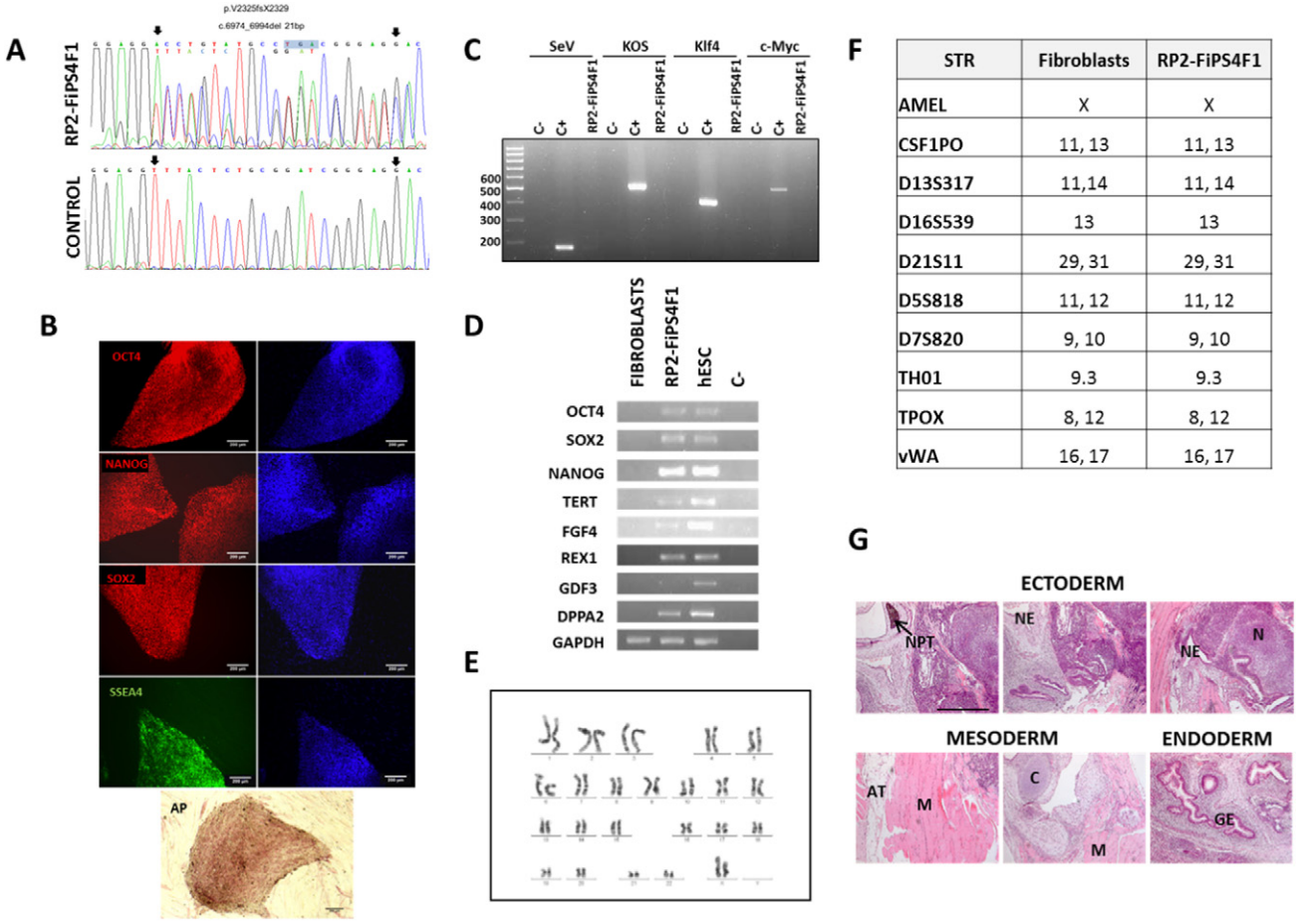

STR	Fibroblasts	RP2-FiPS4F1
AMEL	X	X
CSF1PO	11, 13	11, 13
D13S317	11,14	11, 14
D16S539	13	13
D21S11	29, 31	29, 31
D5S818	11, 12	11, 12
D7S820	9, 10	9, 10
TH01	9.3	9.3
TPOX	8, 12	8, 12
vWA	16, 17	16, 17

Fig. 1. Molecular and functional characterization of the RP2-FiPS4F1 hiPSC line. **A**. Chromatograms showing the sequence around the mutation site (*PRPF8*, c.6974_6994del21bp) in the patient's hiPSC line and in healthy control hiPSC line. The deleted sequence is marked by arrows. The premature stop codon is marked in blue. **B**. Immunofluorescence analysis showing expression of pluripotency markers: transcription factors OCT-4, NANOG, SOX2 and the surface marker SSEA-4. Blue staining: DAPI. AP: Positive alkaline phosphatase staining. Scale bars: 200 μm. **C**. RT-PCR detecting the clearance of the vectors and the exogenous reprogramming factor genes Oct4, Sox2, c-Myc, Klf4. **D**. Semiquantitative RT-PCR showing the expression of the pluripotency associated markers OCT-4, SOX2, NANOG, TERT, FGF4, REX1, GDF3, DPPA2. **E**. Karyotype analysis. RP2-FiPS4F1 has a normal female karyotype (46, XX). **F**. DNA fingerprinting analysis showing the match between RP2-FiPS4F1 cell line and patient's fibroblasts. **G**. *In vivo* differentiation assay (*i.e.* teratoma assay) showing ectodermal (NPT - Neural pigmented tissue; NE - Neuroepithelial; N - Neural), mesodermal (AT - Adipose tissue; C - Cartilage; M - Muscle) and endodermal (GE - gut epithelium) tissue. Scale bar: 500 μm.

injection, tumor was formed and extracted. Histological sections showed that the tumor contained derivates of all three germ layers including gut-like tissues (endoderm), neural cells and retina (ectoderm) and striated muscle, bone, cartilage and adipose tissue (mesoderm) (Fig. 1G).

Materials and methods

Derivation of patient's fibroblasts

A skin biopsy of healthy individual and affected patient (with a confirmed clinical diagnosis of RP) was taken following informed consent. The skin biopsy sample was placed in a 15-ml conical tube containing 15 ml of fibroblast cell culture media for shipment at room temperature (20–25 °C). The biopsy sample was cut into small pieces and cultured in 60-mm culture dishes (8–10 pieces per dish) in DMEM (Invitrogen, #21969-035) containing 10% FBS (Gibco #10270), 2 mM GlutaMAX (Invitrogen, #35050-038), 1× Penicillin-Streptomycin (Invitrogen #15140-163) at 37 °C under 5% CO_2. After 3–4 weeks, fibroblasts outgrowing from the biopsy pieces cover most of the dish and cells were passaged and plated into a T-75 flask (passage 1). The fibroblasts were further expanded for several passages and cryopreserved in liquid nitrogen tank.

Reprogramming patient's fibroblasts

The experimental protocols in the present study were approved by research ethical committee, Commission of Guaranties, according to Spanish legal framework. The hiPSC were derived from patient's fibroblasts using Sendai virus (Cyto Tune-iPS 2.0 reprogramming Kit, Life Technologies) according to manufacturer instructions. hiPSCs were grown on irradiated (45Gy) human foreskin fibroblasts (ATCC CRL 2429) in hiPSCs medium containing KO DMEM, KSR 20%, Glutamax 2 mM, non-essential amino acids 0.1 mM, β-mercaptoethanol 0.23 mM, basic FGF 10 ng/ml, penicillin/streptomycin. Cells were mechanically passaged every 6–8 days.

The elimination of Sendai virus transgenes was detected by RT-PCR at passages 8–10. Total RNA was extracted by RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, and treated with DNase1 to remove any genomic DNA contamination. Cells set aside during reprogramming (day 7) were used as a positive control (C+). The reaction mix without RNA was used as negative control (C−). PCR was carried out by using 500 ng of cDNA in the presence of MyTaq DNA Polymerase (Bioline) with the parameters described by the manual. RT-PCR primer sets used for detecting the SeV genome and transgenes in reprogrammed cells are described in Table 1. The PCR products were analyzed using 2% agarose gel electrophoresis.

Table 1
RT-PCR primer sets used for detecting the SeV genome, SeV transgenes and pluripotency markers.

Primer	Forward 5′3′	Reverse 5′3′	Annealing (°C)	Size (bp)
SeV	GGATCACTAGGTGATATCGAGC	ACCAGACAAGAGTTTAAGAGATATGTATC	55	181
KOS	ATGCACCGCTACGACGTGAGCGC	ACCTTGACAATCCTGATGTGG	55	528
Klf4	TTCCTGCATGCCAGAGGAGCCC	AATGTATCGAAGGTGCTCAA	55	410
c-Myc	TAACTGACTAGCAGGCTTGTCG	TCCACATACAGTCCTGGATGATGATG	55	532
NANOG	CCAAATTCTCCTGCCAGTGAC	CACGTGGTTTCCAAACAAGAAA	60	260
OCT4	AAGCCCTCATTTCACCAGG	CTTGGAAGCTTAGCCAGGTC	60	165
SOX2	TCACATGTCCCAGCACTACC	CCCATTTCCCTCGTTTTTCT	60	137
TERT	TGGCTGCGTGGTGAACTTG	CCAGTCTCACCTTCAACCGC	60	205
FGF4	CTACAACGCCTACGAGTCCTACA	CAACTCTGACTTTTCTGGTGCAAC	60	371
REX1	CAGATCCTAAACAGCTCGCAGAAT	TCTGGACTTTAATTTGCGTACGC	60	306
GDF3	CTTATGCTACGTAAAGGAGCTGGG	AACTTCCGGGACCTGGGTTGGCAC	65	631
DPPA2	CCGTCCCCGCAATCTCCTTCCATC	CACCGGGAGCCATGTTGGCATCAT	65	606
GAPDH	ATCGTGGAAGGACTCATGACCACA	CCCTGTTGCTGTAGCCAAATTCGT	60	463

Teratoma assay

For teratoma assay the colonies from a fully confluent 6-well plate were cut mechanically and resuspended in hiPSCs media and Matrigel (BD, #354277). The suspension was immediately injected into SCID nude mice subcutaneously at the dorsal part. After about 8 weeks, the teratomas of 1 cm diameter were formed and excised. Teratomas were fixed in 4% paraformaldehyde (PFA) for 3 days, washed 3 times with PBS, sectioned in half and observed macroscopically for the presence of cysts. Then the samples were dehydrated using a 70–80–95% ethanol and xylol and embedded in paraffin. Continuous 6 μm cuts were performed.

Karyotype analyses

To analyze genome integrity the hiPSCs were adapted to feeder-free cell culture conditions using mTeSR1 medium. After two passages the hiPSCs were analyzed by G-banding at 400–550 band resolution (Biobanco de Sistema Sanitario Público, Granada, Spain).

Fingerprinting

gDNA form fibroblasts and hiPSCs was extracted using QIAamp DNA Blood mini kit (Quiagen) in the presence of RNAse (Roche). Fingerprinting analyses was performed using Promega kit 10 microsatellite markers (TH01, TPOX, vWA, CSF1PO, D16S539, D7S820, D13S317, D5S818 y D21S11, Amelogenin) and analyzed on Abi PRISM 3130 using GeneMapper (Thermo Fisher) by Biobanco de Sistema Sanitario Público, Granada, Spain.

Mutation sequencing

Genomic DNA from fibroblasts and hiPSCs was isolated using the QIAamp DNA Blood mini kit (Quiagen). Primers used for amplification and directed sequencing of *PRPF8* around the mutation site (c.6974_6994): Sense: 5′-GATAGCAGTAGGGATAAGGTGAG-3′, Antisense: 5′-GCTGAAGCAGGAGGCAGGGAAAC-3′.

Detection of pluripotency markers by RT-PCR

hiPSC colonies were mechanically cut and were collected by centrifugation and total RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, and treated with DNase1 to remove any genomic DNA contamination. QuantiTect Reverse Transcription Kit (Qiagen) was used to carry out cDNA synthesis from 1 μg of total RNA according to the manufacturer's instructions. The expression level of pluripotency markers was analyzed using the primers described in Table 1.

The PCR reaction was performed with MyTaq DNA Polymerase (Bioline GmbH, Luckenwalde, Germany) with denaturation 94 °C, 15 s, annealing 60–65 °C; 30 s, extension 72 °C, 45 s, for 35 cycles. Glyceraldehydes 3-phosphate dehydrogenase (GAPDH) expression was used as a control housekeeping gene. Thereafter, PCR products were analyzed on 2% agarose gels.

Immunocytochemistry

Cells were washed in ice-cold PBS and fixed in 4% PFA for 15 min. Fixed cells were washed twice in PBS and placed in blocking solution (3% normal goat or donkey serum and 0.5% Triton-X100 in PBS) for 1 h at room temperature. Cells were then incubated overnight at 4 °C with one of the following primary antibodies: rabbit anti-NANOG (1:400, Cell Signaling), rabbit anti-OCT4 (1:400, Cell Signaling), rabbit anti-SOX2 (1:400, Cell Signaling), mouse anti-SSEA4 (1:100, BD Pharmigen), The following day, cells were washed three to five times in PBS and incubated with an appropriate secondary antibody (1:500, Invitrogen). After secondary antibody incubation, cells were stained with 4′,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Life Technologies, #D1306) (1:1000), washed three times in PBS. Cells stained in plastic dishes were visualized on Leica DM600 fluorescent microscope equipped with Leica DC500 camera.

Alkaline phosphatase staining

Alkaline phosphatase staining was carried out using Alkaline Phosphatase Staining Kit II (Stemgent, MA).

Mycoplasma detection

The presence of mycoplasma was tested regularly measuring enzyme activity *via* luciferase (MycoAlert™ PLUS Mycoplasma Detection Kit, Lonza) and was in all cases absent.

hiPSC Nomenclature

The generated hiPSC line was named according to Spanish National Stem Cell Bank recommendations. The line is registered on https://hpscreg.eu/ as RCPFi001-A line.

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