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Lab Resource: Stem Cell Line

Generation of a human iPSC line from a patient with congenital glaucoma caused by mutation in CYP1B1 gene

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ABSTRACT

The human iPSC cell line, GLC-FiPS4F1 (ESi047-A), derived from dermal fibroblast from the patient with congenital glaucoma caused by the mutation of the gene *CYP1B1*, was generated by non-integrative reprogramming technology using OCT3/4, SOX2, CMYC and KLF4 reprogramming factors.

Resource table

Unique stem cell line identifier	ESi047-A
Alternative name(s) of stem cell line	GLC-FiPS4F1
Institution	Research Center Principe Felipe, Eduardo Primo Yufera 3, Valencia, Spain
Contact information of distributor	Slaven Erceg, serceg@cipf.es
Type of cell line	iPSC
Origin	Human
Additional origin info	Age:38 Sex: Male
Cell Source	Dermal fibroblasts
Clonality	Clonal
Method of reprogramming	Sendai virus
Genetic Modification	No
Type of Modification	n/a
Associated disease	Congenital glaucoma
Gene/locus	Gene: cytochrome P450 family 1 subfamily B member 1 (CYP1B1) Locus: 2p22.2 Mutation: c.1403_1429dup (p.K468_S476dup) homozygosis
Method of modification	n/a
Name of transgene or resistance	n/a
Inducible/constitutive system	n/a
Date archived/stock date	n/a
Cell line repository/bank	Spanish Stem Cell Bank
Ethical approval	Patient informed consent obtained/Ethics Review Board-competent authority approval obtained

Resource utility

The generation of induced pluripotent stem (iPS) cells from the glaucoma patients permits the development of disease specific stem cells that can be further differentiated toward affected cells to better understand molecular mechanisms of disease and pathophysiology.

Resource details

Skin punch biopsy was taken from a 38-year-old patient who was diagnosed with congenital glaucoma (GLC) harboring c.1403_1429dup (p.K468_S476dup) mutation in homozygosis in cytochrome P450 family 1 subfamily B member 1 (CYP1B1) gene and primary fibroblast cell line was established. The generation of the human induced pluripotent

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stem cell (hiPSC) line, GLC-FiPS4F1 (registered as ESi047-A at www.hPSCreg.com), was carried out using non-integrative Sendai virus containing the human reprogramming factors, Oct3/4, Sox2, C-Myc, Klf4 (Takahashi et al., 2007) 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 GLC-FiPS4F1 confirmed the mutation in both alleles in CYP1B1 (Fig. 1A). Pluripotency was additionally assessed by immunocytochemistry to pluripotency markers OCT-4, SOX2, NANOG and SSEA-4 and flow cytometry for SSEA-4 pluripotency marker (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 (Fig. 1B). The clearance of the virus and the exogenous reprogramming factor genes was confirmed by RT-PCR after twelve cell culture passages (Fig. 1C). The expression of endogenous plutipotency genes was detected by RT-PCR (Fig. 1D). The genetic fingerprinting was performed with GLC-FiPS4F1 hiPSC line and proved its genetic identity to parental fibroblasts (submitted in archive with journal). The selected line was karyotypicaly normal at low and medium passages (Fig. 1E). The mycoplasma was regularly checked without positive results. To test the ability of the hiPSC line to generate derivates of three germ layers *in vitro*, the hiPSC were differentiated using an embryoid body based assay. Spontaneous differentiated cells were immunostained for differentiation markers such as PAX6 and TUBB for ectoderm, CS for mesoderm and positive AFP for endoderm (Fig. 1F).

Materials and methods

Reprogramming patient's fibroblasts

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 passage 12. Total RNA was extracted by RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, and

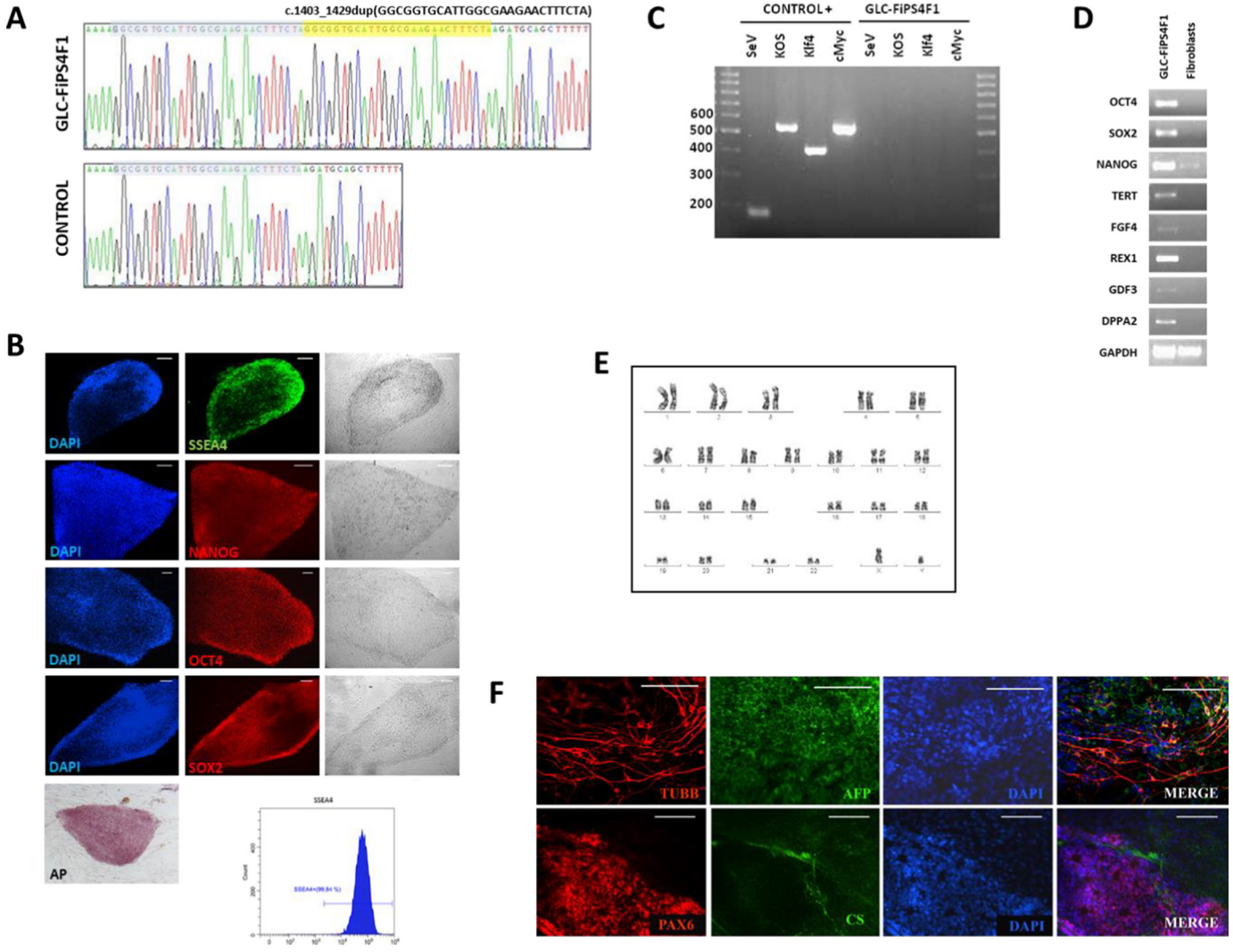

Fig. 1. Molecular and functional characterization of the GLC-FiPS4F1 hiPSC line. A. Chromatograms showing the sequence around the mutation site in *CYP1B1* gene in the patient's hiPSC line and in healthy control hiPSC line. Change in sequence (c.1403_1429dup) is marked in yellow. B. Immunofluorescence analysis showing expression of pluripotency markers: transcription factors SSEA, NANOG, OCT-4 and SOX2 (Scale bars 500 μm). Additional graph of FACS analysis for SSEA4 marker. AP: Positive alkaline phosphatase staining. 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. GLC-FiPS4F1 has a normal male karyotype (46, XY). F. In vitro differentiation assay (EB assay) showing ectodermal (TUJ1 and PAX6), mesodermal (CS; chondroitin sulphate) and endodermal (AFP; Alpha-fetoprotein) markers. Scale bar: 500 μm.

treated with DNase I to remove any genomic DNA contamination. Cells set aside during reprogramming (day 7) were used as a positive 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.

In vitro differentiation assay

For *in vitro* differentiation assay the colonies from a fully confluent 6-well plate were cut mechanically and cultured in suspension to form embryoid bodies in hiPSCs media without bFGF. After 7 days in suspension, embryoid bodies were transferred into 0.1% gelatin-coated plates and cultured for additional 7–10 days to allow spontaneous differentiation. Then the cells were fixed and immunostained to detect cells from the three germ layers.

Karyotype analysis

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 (Qiagen) 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 (Qiagen). Primers used for amplification and directed sequencing of *CYP1B1* around the mutation site (c.1403_1429): Sense: 5′-CAACCTGCCCTATGTCCTGG-3′, Antisense: 5′-GGGTATGGAGCACACCTCAC-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 DNase I 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 2.

The PCR reaction was performed with MyTaq DNA Polymerase (Bioline GmbH, Luckenwalde, Germany) with following steps: denaturation 94 °C; 15 s, annealing 60–65 °C; 30 s, extension 72 °C; 45 s, for 35 cycles. Glyceraldehyde 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), rabbit anti-PAX6 (1:500, Covance), mouse anti-chondroitin sulfate (CS) (1:200, abcam), rabbit anti-neuron-specific class III beta-tubulin (TUBB) (1:100, Covance) and mouse anti-alpha fetoprotein (AFP) (1:100, R&D systems). 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 DAPI (1:1000), washed three times in PBS. Cells stained in plastic dishes were visualized on Leica DM600 fluorescent microscope equipped with Leica DC500 camera.

Flow cytometry

hiPSCs were dissociated using accutase (Innovative Cell Technologies) and expression of pluripotency marker (SSEA-4) was analyzed using a CytoFLEX flow cytometer (Beckman Coulter).

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Shown by immunocytochemistry
Phenotype	Immunocytochemisty	Assess staining/expression of pluripotency markers: Oct4, Nanog, Sox2, SSEA4	Fig. 1 panel B
	Cytometry	SSEA4 99,84%	Fig. 1 panel B
Genotype	Karyotype (G-banding) and resolution	46XY, Resolution 450–500	Fig. 1 panel E
Identity	Microsatellite PCR (mPCR)	Not performed	
	STR analysis	10 loci analyzed, all matching	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	Homozygous type of mutation	Fig. 1 panel A
	Southern Blot OR WGS	No	
Microbiology and virology	Mycoplasma	Mycoplasma testing by luminescence. Negative	Performed but not shown
Differentiation potential	Embryoid body formation	Positive PAX6 and TUBB ectodermal staining, positive CS mesodermal staining and positive AFP endodermal staining.	Fig. 1 panel F
Donor screening (OPTIONAL)	N/A	N/A	N/A
Genotype additional info (OPTIONAL)			

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Rabbit anti-Nanog	1:400	Cell Signaling Technology Cat# D73G4
Pluripotency markers	Rabbit anti-Oct4	1:400	Cell Signaling Technology Cat# C30A3
Pluripotency markers	Rabbit anti-Sox2	1:400	Cell Signaling Technology Cat# D6D9
Pluripotency markers	Rabbit anti-SSEA4	1:100	BD Pharmigen Cat# 560073
Pluripotency markers	Mouse anti human SSEA4-PE	1:800	STEMCELL Technologies Cat# 60062PE.1
Differentiation markers	Rabbit anti-PAX6	1:500	Covance Cat# PRB-278P
Differentiation markers	Mouse anti-CS	1:200	Abcam Cat# ab11570
Differentiation markers	Mouse anti-AFP	1:100	R&D Cat# MAB1368
Differentiation markers	Mouse anti-BTubulin (Tuj1)	1:500	Neuromics Cat# MO15013

Primers		
	Target	Forward/Reverse primer (5′-3′)
Pluripotency markers (qPCR)	OCT4	AAGCCCTCATTTCACCAGG
		CTTGGAAGCTTAGCCAGGTC
Pluripotency markers (qPCR)	NANOG	CCAAATTCTCCTGCCAGTGAC
		CACGTGGTTTCCAAACAAGAAA
Pluripotency markers (qPCR)	SOX2	TCACATGTCCCAGCACTACC
		CCCATTTCCCTCGTTTTTCT
Pluripotency markers (qPCR)	TERT	TGGCTGCGTGGTGAACTTG
		GCGGTTGAAGGTGAGACTGG
Pluripotency markers (qPCR)	FGF4	CTACAACGCCTACGAGTCCTACA
		GTTGCACCAGAAAAGTCAGAGTTG
Pluripotency markers (qPCR)	REX1	CAGATCCTAAACAGCTCGCAGAAT
		GCGTACGCAAATTAAAGTCCAGA
Pluripotency markers (qPCR)	GDF3	CTTATGCTACGTAAAGGAGCTGGG
		GTGCCAACCCAGGTCCCGGAAGTT
Pluripotency markers (qPCR)	DPPA2	CCGTCCCCGCAATCTCCTTCCATC
		ATGATGCCAACATGGCTCCCGGTG
House-keeping gene (qPCR)	GAPDH	ATCGTGGAAGGACTCATGACCACA
		CCCTGTTGCTGTAGCCAAATTCGT
Sendai virus detection	SeV	GGATCACTAGGTGATATCGAGC
		ACCAGACAAGAGTTTAAGAGATATGTATC
Transgenes detection	KOS	ATGCACCGCTACGACGTGAGCGC
		ACCTTGACAATCCTGATGTGG
Transgenes detection	c-Myc	TAACTGACTAGCAGGCTTGTCG
		TCCACATACAGTCCTGGATGATGATG
Transgenes detection	Klf4	TTCCTGCATGCCAGAGGAGCCC
		AATGTATCGAAGGTGCTCAA

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 following Spanish National Stem Cell Bank recommendations. The line is registered at https://hpscreg.eu/ as ESi047-A line.

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Reference

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