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Lab resource: Stem Cell Line

Generation of a human iPS cell line from a patient with retinitis pigmentosa due to EYS mutation

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A B S T R A C T

Retinitis pigmentosa (RP) is an inherited retinal degenerative disease. Mutations in *EYS* have been associated with autosomal recessive RP. The human iPS cell line, CABi002-A, derived from peripheral blood mononuclear cells from a patient carrying a heterozygous double mutation in *EYS* gene was generated by non-integrative reprogramming technology, using hOCT3/4, hSOX2, hc-MYC and hKLF4 reprogramming factors. Pluripotency and differentiation capacity were assessed by immunocytochemistry and RT-PCR. This iPSC line can be further differentiated towards the affected cells to understand the pathophysiology of the disease and test new therapeutic strategies.

Resource table

Unique stem cell line identifier	CABi002-A
Alternative name(s) of stem cell line	OF0176-EYS02-C7
Institution	Andalusian Molecular Biology and Regenerative Medicine Centre (CABIMER)
Contact information of distributor	Francisco Diaz-Corrales, francisco.diaz@cabimer.es
Type of cell line	iPSC
Origin	Human
Additional origin info	Age:38 Sex: Male Ethnicity if known: Caucasian
Cell source	Peripheral blood mononucleated cells
Clonality	Clonal
Method of reprogramming	Sendai viral reprogramming
Genetic modification	No
Type of modification	N/A
Associated disease	Autosomal Recessive Retinitis Pigmentosa
Gene/locus	EYS/6q12 c.3567delA c.4829-4832delCATT
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	N/A
Ethical approval	Patient informed consent obtained/Ethics Review Board-competent authority approval obtained (Ethical Approval number: PR-01-2015)

Resource utility

The generation of this cellular model will allow us to better understand the pathophysiology of the disease and to test new therapeutic strategies for RP due to *EYS* mutations.

Resource details

Mononucleated cells were collected from 4 ml of peripheral blood sample from 38 year-old patient diagnosed with inherited con-rod dystrophy, due to an heterozygous double mutation in *EYS* gene caused by a deletion of an A in the position 3567 of the exon 23 of the paternal allele (c.3567delA p.Gly1190Aspfs*39), originating a frame reading change and premature STOP codon and a deletion of a CATT in the position 4829–4832 of the exon 26 of the maternal allele (c.4829-

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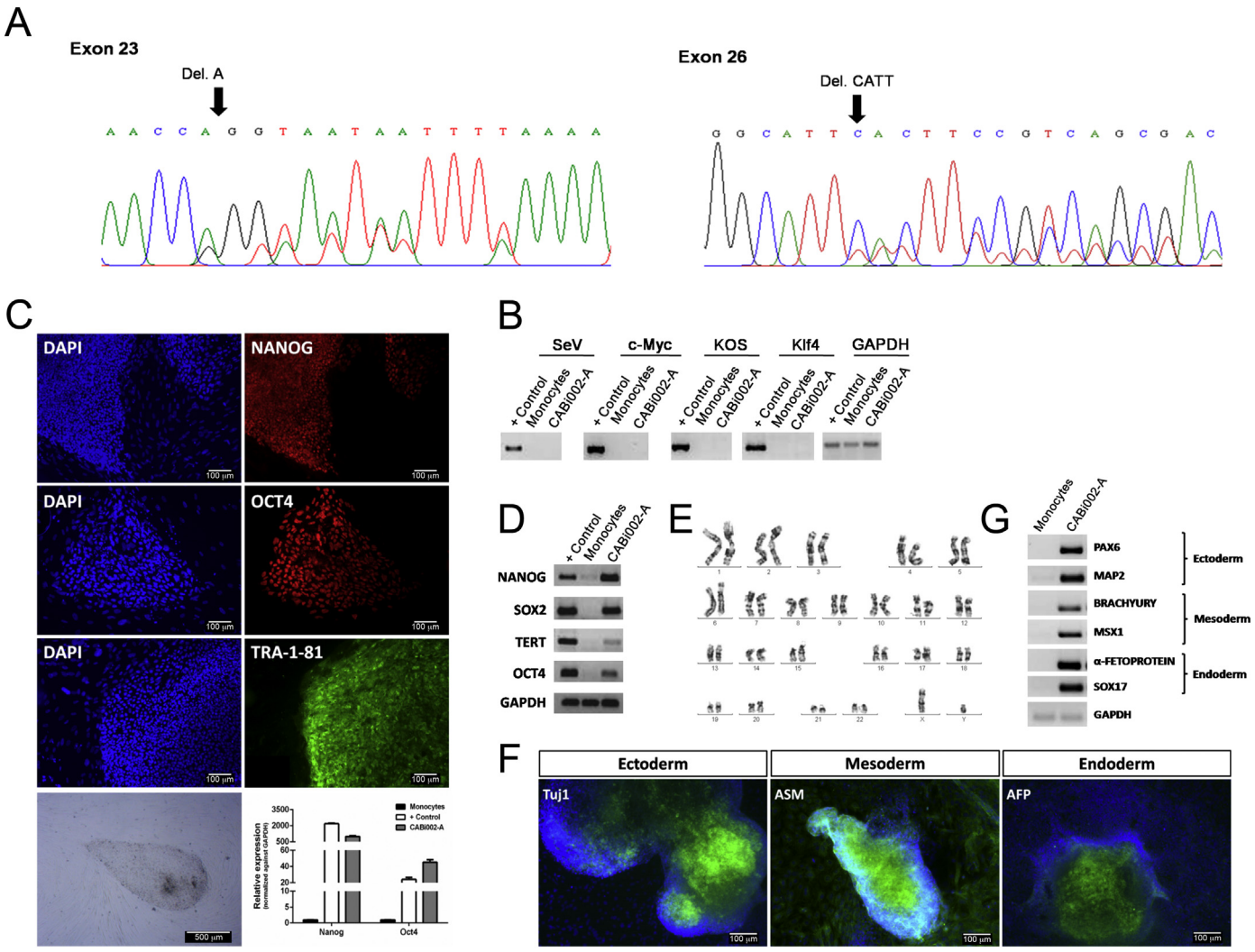

Fig. 1. Characterization of CABi002-A line. A. DNA electropherograms showing the heterozygous double mutation c.3567delA and c.4829-4832delCATT of *EYS* gene in CABi002-A hiPS cell line of a RP patient. B. RT-PCR analysis of SeV genome and transgenes in hiPSCs, PBMC's (monocytes) and positive control (+ Control). C. Immunocytochemistry for pluripotency markers NANOG, OCT4 and TRA-1-81. Nuclei were counterstained with DAPI. Lower panel (left) is a brightfield image of a CABi002-A hiPS colony, showing its normal morphology and (right) quantification by qPCR of pluripotency markers *NANOG* and *OCT*3/4 of CABi002-A hiPS cells compared, compared to PBMC's (monocytes). D. RT-PCR analysis of pluripotency markers. E. Representative metaphase of normal human karyotype (46, XY). F. Immunocytochemistry for ectodermal (Tuj1), mesodermal (ASM) and endodermal (AFP) germ layer markers with nuclei counterstained with DAPI. G. RT-PCR analysis of the three germ layer markers.

4832delCATT p.Ser1610Phefs*7) (McGuigan et al., 2017). DNA sequencing of CABi002-A confirmed the presence of the aforementioned mutations (Fig. 1A). The human induced pluripotent stem (hiPS) cell line was generated by using Sendai virus, encoding the reprogramming factors hOCT3/4, hc-MYC, hKLF4, and hSOX2 (Takahashi et al., 2007), according to manufacturer's instructions.

The obtained colonies presented stem-like morphology and pluripotency markers Nanog, Oct4, and TRA-1-81 staining (Fig. 1C). The clearance of viral and endogenous reprogramming markers (Fig. 1B), as well as the presence of pluripotency genes (Fig. 1D), was confirmed by RT-PCR after eight cell culture passages. Our results on the karyotype analysis showed that CABi002-A cell line exhibited a normal, diploid (46, XY) chromosomal content (Fig. 1E) and the genetic fingerprinting proved the genetic identity to parental mononucleated blood cells (archived with journal). Pluripotency was tested by the ability of CABi002-A to generate the three germ layers *in vitro*: endoderm, mesoderm and ectoderm, as confirmed by immunofluorescent staining of α-fetoprotein (AFP), vimentin and III beta-tubulin (Tuj1), respectively (Fig. 1F) and RT-PCR (Fig. 1G).

Materials and methods

Mutation sequencing

Genomic DNA from peripheral blood mononuclear cells (PBMCs) and hiPSCs was isolated using the QIAamp DNA Blood mini kit (Qiagen). Primers used for amplification and directed sequencing of *EYS* flanking the mutation sites are described in Table 1.

Reprogramming of PBMCs

PBMCs were isolated using the Vacutainer® CPT™ tubes (BD Biosciences). PBMCs were cultured (1.0×10^6 cells) in Expansion Medium (EM; QBSF-60 medium; 50 µg/ml ascorbic acid, 1% Pen/Strep, 50 ng/ml SCF, 10 ng/ml IL-3, 2 U/ml EPO, 40 ng/ml IGF-1 and 1 µM Dexamethasone), for one week before transduction with CytoTune®-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific). Briefly, 0.25×10^5 cells were transduced using MOI of 5–5-3 (hKOS, hc-MYC, hKLF4, respectively). After 24 h of incubation cells were collected, centrifuged and seeded in a 24 well plate containing EM. Two days later

Table 1
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Rabbit anti-OCT4	1:400	Cell Signaling Technology Cat# 2840, RRID:AB_2167691
	Rabbit anti-NANOG	1:400	Cell Signaling Technology Cat# 4903, RRID:AB_10559205
	Rabbit anti-SOX2	1:400	Cell Signaling Technology Cat# 3579, RRID:AB_2195767
	Mouse anti-SSEA-4	1:100	BD Biosciences Cat# 560073, RRID:AB_1645601
	Mouse anti-TRA-1-81	1:100	Stemgent Cat# 09-0069, RRID:AB_2119069
Differentiation markers	Rabbit anti- Tuj1	1:2000	Covance Research Products Inc. Cat# MRB-435P-100, RRID:AB_663339
	Mouse anti-ASM	1:300	Sigma-Aldrich Cat# A5228, RRID:AB_262054
	Mouse anti-AFP	1:20	Sigma-Aldrich Cat# A5228, RRID:AB_262054
Secondary antibodies	Donkey anti-Mouse 488	1:500	Molecular Probes Cat# A-21202, RRID:AB_141607
	Donkey anti-Rabbit 594	1:500	Molecular Probes Cat# A-21207, RRID:AB_141637

Primers		
	Target	Forward/Reverse primer (5′-3′)
Plasmids (RT-PCR)	SeV plasmid	GGATCACTAGGTGATATCGAGC/ACCAGACAAGAGTTTAAGAGATATGTATC
	KOS plasmid	ATGCACCGCTACGACGTGAGCGC/ACCTTGACAATCCTGATGTGG
	KLF4 plasmid	TTCCTGCATGCCAGAGGAGCCC/AATGTATCGAAGGTGCTCAA
	c-MYC plasmid	TAACTGACTAGCAGGCTTGTCG/TCCACATACAGTCCTGGATGATGATG
Pluripotency markers (RT-PCR)	NANOG	CCAAATTCTCCTGCCAGTGAC/CACGTGGTTTCCAAACAAGAAA
	OCT4	AAGCCCTCATTTCACCAGG/CTTGGAAGCTTAGCCAGGTC
	SOX2	TCACATGTCCCAGCACTACC/CCCATTTCCCTCGTTTTTCT
	TERT	GCGTTTGGTGGATGATTTCT/GGCATAGCTGGAGTAGTCGC
Differentiation Potential (RT-PCR)	PAX6	GTCCATCTTTGCTTGGGAAA/TAGCCAGGTTGCGAAGAACT
	MAP2	GCACGCCTGCAGCTTGCATC/TCTCCACCACCCCGTACGCA
	BRACHYURY	TCAGCAAAGTCAAGCTCACCA/CCCCAACTCTCACTATGTGGATT
	MSX1	CGAGAGGACCCCGTGGATGCAGAG/GGCGGCCATCTTCAGCTTCTCCAG
	α-FETOPROTEIN	CTTTGGGCTGCTCGCTATGA/ATGGCTTGGAAAGTTCGGGTC
	SOX17	CGCTTTCATGGTGTGGGCTAAGGACG/TAGTTGGGGTGGTCCTGCATGTGCTG
House-Keeping genes (RT-PCR)	GAPDH	TGCACCACCAACTGCTTAGC/GGCATGGACTGTGGTCATGAG
Genotyping	EYS exon 23	TCCCAGCTACATGTTGTTG/CATTAAGATTTCCTGATGAAAGC
	EYS exon 26	CAAGCAACCAGAGACTCA/TGTGAAGGGACAATGGATAAAC

0.1×10^6 cells were transferred onto a 6 well plate covered with 0.25×10^6 irradiated human foetal fibroblasts (irHFF) in QBSF-60 medium, supplemented with 50 μg/ml ascorbic acid and 1% Pen/Strep. Seven days post-transduction, culture medium was replaced by iPS medium (KO DMEM, 20% KO serum, 1% GlutaMAX; 1% MEM NEAA, 0.23 mM β-mercaptoethanol, 1% Pen/Strep, 10 ng/ml bFGF). Individual colonies with stem-like morphology were manually isolated and expanded 21 to 27 days post-transduction. hiPS cell cultures were cultured on 6 well plate coated 0.25×10^6 irHFF, maintained at 37 °C in humidified atmosphere containing 5% CO_2, and passed every week.

RT-PCR for detection of viral clearance and pluripotency markers

Total RNA was isolated from cultured hiPS cells with RNeasy Mini Kit (Qiagen) and treated with DNase1 to remove genomic DNA contamination. 1 μg of total RNA was used as template to obtain cDNA, using QuantiTect Reverse Transcription Kit (Qiagen). Viral clearance and pluripotency markers detection were analyzed using the primers described in Table 1. RT-PCR reaction was performed using MyTaq DNA Polymerase (Bioline GmbH). PCR products were analyzed on 2% agarose gels.

Three linage differentiation

In vitro differentiation was performed by embryoid body (EB) formation to generate the three germ layers (endoderm, mesoderm and ectoderm). The hiPS cells were separated manually from the feeder cells and cultured in non-adherent conditions in iPS medium without bFGF (KO DMEM, 20% KO serum, 1% GlutaMAX; 1% MEM NEAA, 0.23 mM β-mercaptoethanol, 1% Pen/Strep) for the following 7 days. Then, the EBs were seeded on glass coverslips treated with 0,1% gelatin for 2 h/RT and cultured during one week in EBs medium (DMEM/F12, 10% FBS, 1% GlutaMAX, 1% MEM NEAA, 1% Pen/Strep). The coverslips were fixed in 4% PFA for 15 min and analyzed by immunofluorescence.

Immunocytochemistry

Cells were allowed to grow in glass coverslips coated with irHFF and washed in ice-cold PBS before fixation in 4% PFA, for 15 min. Fixed cells were washed twice in PBS and placed in blocking solution (2% donkey serum in 0.2% Triton-X100/PBS) for 1 h at room temperature. Cells were incubated for 1 h at room temperature with the primary antibody (Table 1). After incubation, samples were washed 3 times in 0.2% Triton X100/PBS, and incubated with the secondary antibodies at room temperature for 1 h (Table 1). After 3 washes, coverslips were mounted with Vectashield mounting medium (Vector H-1200) containing 4,6-diamidino-2-phenylindole (DAPI).

Karyotype analyses

Genome integrity of the hiPS cells was analyzed by G-banding at 400–550 band resolution (Biobanco de Sistema Sanitario Público, Granada, Spain).

Fingerprinting

gDNA form PBMC's and hiPS cells was extracted using QIAamp DNA Blood mini kit (Qiagen) in the presence of RNAse (Roche). Fingerprinting analyses was performed by Biobanco de Sistema Sanitario Público, Granada, Spain.

Mycoplasma detection

The presence of mycoplasma was tested regularly by luminescence using the MycoAlert™ PLUS Mycoplasma Detection Kit (Lonza).

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