Stem Cell Research 38 (2019) 101455

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Lab Resource: Multiple Cell Lines

Generation of an iPSC line from a retinitis pigmentosa patient carrying a homozygous mutation in *CERKL* and a healthy sibling

Arantxa Bolinches-Amorós[a], Marian León[a], Verónica del Buey Furió[a], Gemma Marfany[b,c], Roser Gonzàlez-Duarte[b,c], Slaven Erceg[a,d], Dunja Lukovic[a,e,*]

[a] National Stem Cell Bank-Valencia Node, Proteomics, Genotyping and Cell Line Platform, PRB3, ISCIII, Research Centre Principe Felipe, c/ Eduardo Primo Yúfera 3, 46012, Valencia, Spain
[b] Dept. de Genètica, Microbiologia I Estadística, Facultat de Biologia, Universitat de Barcelona, CIBERER, IBUB-IRSJD, Avda. Diagonal 643, 08028 Barcelona, Spain
[c] DBGen Ocular Genomics, Barcelona, Spain
[d] Stem Cells Therapies in Neurodegenerative Diseases Lab, Research Center Principe Felipe, c/Eduardo Primo Yúfera 3, 46012, Valencia, Spain
[e] Retinal Degeneration Lab, Research Centre Principe Felipe, c/ Eduardo Primo Yúfera 3, 46012, Valencia, Spain

A B S T R A C T

Dermal fibroblasts from an autosomal recessive retinitis pigmentosa (RP) patient, homozygous for the mutation c.769 C > T, p.Arg257Ter, in *CERKL* (*Ceramide Kinase-Like*) gene, and a healthy sibling were derived and reprogrammed by Sendai virus. The generated human induced pluripotent stem cell (hiPSC) lines RP3-FiPS4F1 and Ctrl3-FiPS4F1, were free of genomically integrated reprogramming genes, showed stable karyotypes, expressed pluripotency markers and could be differentiated towards the three germ layers in vitro. These hiPSC lines offer a useful resource to study RP pathomechanisms, drug testing and therapeutic opportunities.

Resource table

Unique stem cell lines identifier	ESi066-A
	ESi067-A
Alternative names of stem cell lines	RP3-FiPS4F1 (ESi066-A), Ctrl3-FiPS4F1 (ESi067-A)
Institution	Research Center Principe Felipe, Valencia, Spain
Contact information of distributor	Dunja Lukovic dlukovic@cipf.es
Type of cell lines	iPSC
Origin	Human
Cell Source	Dermal fibroblasts
Clonality	Clonal
Method of reprogramming	Sendai virus
Multiline rationale	Disease and healthy sibling control
Gene modification	Yes
Type of modification	Hereditary
Associated disease	Retinitis pigmentosa
Gene/locus	*CERKL* (NM_201548.4), Chr2:g.182423344 G > A (hg19); Ex 5. c.[769 C > T] (p.Arg257Ter)
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	November 5, 2018
Cell line repository/bank	http://www.isciii.es/ISCIII/es/contenidos/fd-el-instituto/fd-organizacion/fd-estructura-directiva/fd-subdireccion-general-investigacion-terapia-celular-medicina-regenerativa/fd-centros-unidades/banco-nacional-lineas-celulares.shtml
Ethical approval	Ethical approval obtained from Valencian Authority for Stem Cell Reseach DGIITC/AMA/pe S-0081/18

Resource utility

Retinitis pigmentosa (RP), a group of inherited retinal dystrophies with a worldwide prevalence of 1:4000, is characterized by photoreceptor degeneration (first rods and later cones) leading to genetic blindness (reviewed in Verbakel et al., 2018). We have generated hiPSC lines from a RP patient homozygous for a nonsense mutation in *CERKL* (p.Arg257Ter), an autosomal recessive RP gene (Tuson et al., 2004). This mutation (also listed as p.Arg283Ter, NM_001030311.2) is the most prevalent *CERKL* mutation in the Spanish population (Ávila-Fernandez et al., 2008). The patient's non-affected sister fibroblasts were also reprogrammed into hiPSCs as a proper control to minimize

* Corresponding author at: Retinal Degeneration Lab and National Stem Cell Bank-Valencia Node, Proteomics, Genotyping and Cell Line Platform, PRB3, ISCIII, Research Centre Principe Felipe, c/ Eduardo Primo Yúfera 3, 46012, Valencia, Spain
E-mail address: dlukovic@cipf.es (D. Lukovic).

https://doi.org/10.1016/j.scr.2019.101455
Received 10 March 2019; Received in revised form 24 April 2019; Accepted 29 April 2019
Available online 04 May 2019

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
RP3-FiPS4F1 (ESi066-A)	RP	Male	62	Caucasian	*CERKL* (NM_201548.4), Chr2:g.182423344 G > A (hg19); Ex 5. c.[769 C > T] (p.Arg257Ter)	Retinitis pigmentosa
Ctrl3-FiPS4F1 (ESi067-A)	Healthy	Female	52	Caucasian	*CERKL* (NM_201548.4) WT	Healthy

the contribution of genetic background to disease phenotype. The non-integrative reprogramming strategy was applied in order to avoid insertional mutations and generate footprint-free hiPSCs that do not contain detectable vectors or transgenes. The cell lines offer a useful resource for RP pathomechanism studies and drug screening.

Resource details

Dermal fibroblast primary cell lines were derived from skin biopsies from a retinitis pigmentosa (RP) patient and his non-affected sister. Fibroblasts were reprogrammed using Sendai virus expressing human OCT4, SOX, KLF4, c-MYC reprogramming factors (Cytotune 2.0; Thermo Fisher). Clonal hiPSC lines from each individual were established and further characterized (Table 1 and Table 2). Sanger sequencing confirmed that the RP patient's fibroblasts (data not shown) and hiPSC line, RP3-FiPS4F1, harboured a nonsense truncating mutation in *CERKL* in homozygosity (Fig. 1A, lower panel), in accordance with previous genotyping data (Tuson et al., 2004). The hiPSCs derived from the healthy sibling, Ctrl3-FiPS4F1, showed the WT sequence in the same region (Fig. 1A, upper panel). Both hiPSC lines showed typical human embryonic stem cell (hESC) –like colony morphology by phase contrast microscopy (Fig. 1B) and were positive for alkaline phosphatase (AP) (Fig. 1C). The expression of pluripotency markers was demonstrated by flow cytometry (SSEA-4 and TRA-1-60) (Fig. 1D), immunofluorescence analyses of OCT4, SOX2, NANOG, SSEA-4 and TRA-1-81 (insets show nuclei staining with DAPI) (Fig. 1E), and RT-PCR for pluripotency markers (Fig. 1F). DNA fingerprinting was used to confirm the origin of the hiPSC clones (Available with authors). Sendai virus was eliminated from the hiPSCs' genome at passage 11 (Supplementary Fig. S1A). To explore genomic integrity, karyotyping was performed and showed that hiPSC lines, RP3-FiPS4F1 and Ctrl3-FiPS4F1, were male and female clones, respectively, and presented normal diploid karyotypes (Supplementary Fig. S1B). The capacity of hiPSCs to differentiate into three germ layers was confirmed by the embryoid body (EB) assay and expression of ectoderm (TUJ1, PAX6), endoderm (AFP, FOXA2) and mesoderm (VIMENTIN, α-SMA) assessed in differentiating EB cultures (Fig. 1G). Mycoplasma was regularly tested throughout the cell culture and was negative (Supplementary Fig. S1C).

Materials and methods

Reprogramming fibroblasts

The primary fibroblasts derived from the skin biopsies were expanded for four passages in DMEM (10% FBS, 2 mM Glutamax, Penicillin-Streptomycin 1 ×) at 37 °C under 5% CO_2, before being reprogrammed by a Sendai virus containing four genes: OCT3/4, SOX2, KLF4 and c-MYC (Cytotune 2.0, Thermo Fisher) according to manufacturer's instructions. hiPSCs were grown on irradiated (45Gy) human foreskin fibroblasts (ATCC CRL 2429) in hiPSC medium containing Knockout-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, at 37 °C/5% CO_2. The hiPSCs were adapted to feeder-free cell culture on plates coated with Matrigel (BD, #354277) using mTeSR1 medium. Matrigel was diluted in DMEM/F-12 according to the dilution factor specified on the certificate of analysis and incubated for 1 h/RT at 1 ml/well of a 6-well plate. Passages were performed using Dispase (STEMCELL Technologies, #07913), every 5–7 days at 1:6–1:10 split ratio.

In vitro differentiation assay

In vitro differentiation was performed by EB formation, namely the hiPSC colonies were lifted manually and cultured in non-adherent conditions in mTeSR1 medium for 24 h, followed by endoderm medium (Knockout-DMEM, 10% FBS, 1% Glutamax, NEAA 1%, β-mercaptoethanol 0.23 mM, Penicillin-Streptomycin 1 ×) for the following 6 days. Thereafter, the EBs were seeded on glass coverslips treated with

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel B
Phenotype	Qualitative analysis (Immunocytochemistry)	Positive staining/expression of pluripotency markers: OCT4, NANOG, SOX2, SSEA-4, TRA-1-81	Fig. 1 panel E
	Quantitative analysis	Healthy: SSEA-4 98,61% TRA-1-60 78,6% RP: SSEA-4 98,87% TRA-1-60 83,7%	. Fig. 1 panel D
Genotype	Karyotype (G-banding) and resolution	Healthy: 46XX; RP: 46XY *Resolution 450–500*	*Supplementary fig. S1B*
Identity	Microsatellite PCR (mPCR) OR STR analysis	*N/A* *10 STR loci analyzed, all matching*	*N/A* *Available with authors*
Mutation analysis (if applicable)	Sequencing	*CERKL (NM_201548.4) ex 5. [c.769 C > T]*	Fig. 1*panel A*
	Southern Blot OR WGS	*N/A*	*N/A*
Microbiology and virology	Mycoplasma	*Mycoplasma testing by luminescence. Negative.*	*Supplementary fig. S1C*
Differentiation potential	Embryoid body formation	*Positive TUJ1 and PAX6 for ectoderm, positive α-SMA, VIMENTIN mesodermal staining and positive AFP and FOXA2 endodermal staining*	Fig. 1*panel G*
Donor screening (optional)	HIV 1 + 2 Hepatitis B, Hepatitis C	*N/A*	*N/A*
Genotype additional info (optional)	Blood group genotyping	*N/A*	*N/A*
	HLA tissue typing	*N/A*	*N/A*

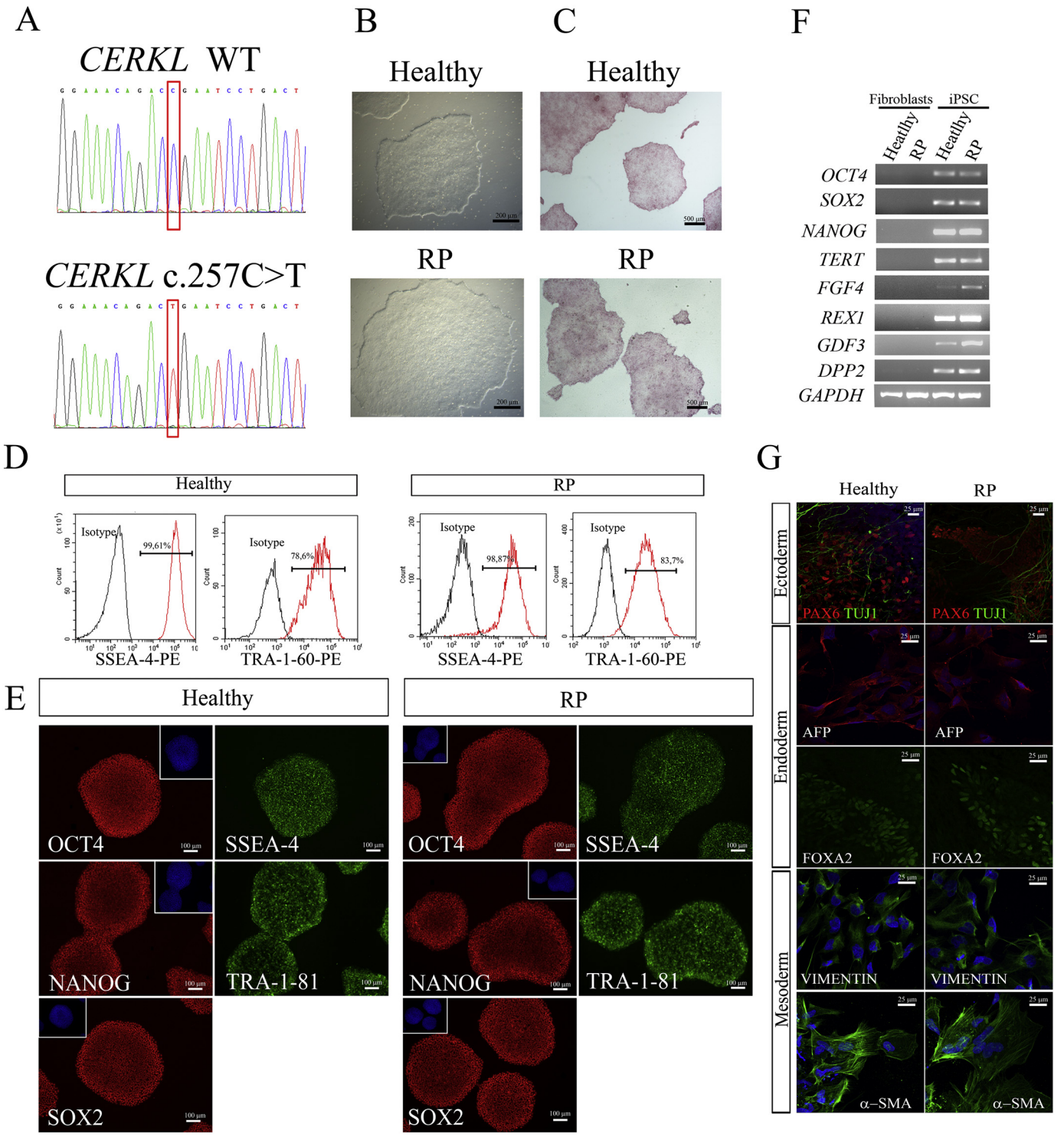

Fig. 1. Characterization of Healthy and RP hiPSC lines. A. DNA sequence electropherograms showing the wild type sequence and the c.257C > T mutation in CERKL in Healthy (upper panel) and RP (lower panel) hiPSC lines. B. Phase contrast micrographs of Healthy and RP hiPSC colonies cultured in feeder-free conditions. C. Alkaline phosphatase positive staining. D. Flow cytometry of surface pluripotency markers SSEA-4 and TRA-1-60. E. Immunofluorescence for pluripotency markers OCT4, SOX2, NANOG, SSEA-4, TRA-1-81. Nuclei were counterstained with DAPI (blue). F. RT-PCR analysis of pluripotency genes in Healthy and RP hiPSC lines. G. Immunocytochemistry for ectodermal (PAX6, TUJ1), endodermal (AFP, FOXA2) and mesodermal (SMA, VITRONECTIN) markers. Nuclei were counterstained with DAPI (blue).

0.1% gelatin for 2 h/RT and cultured during 2 weeks in three cell culture media: ectoderm medium (50% Neurobasal medium, 50% DMEM/F12, 1% N2 supplement, 0.5% B27 supplement, penicillin-streptomycin 1 ×), endoderm medium and mesoderm medium (endoderm medium, 0.5 mM ascorbic acid). The coverslips were fixed with 4% PFA for 15 min and analyzed by immunofluorescence (Table 3). Confocal images were taken with a Leica SP8 microscope.

Table 3
Reagent details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency marker	*Rabbit anti-NANOG*	*1:400*	*Cell Signaling Technology Cat# D73G4 #RRID:AB_10559205*
Pluripotency marker	*Rabbit anti-OCT4*	*1:400*	*Cell Signaling Technology Cat# C30A3 #RRID:AB_2167691*
Pluripotency marker	*Rabbit anti-SOX2*	*1:400*	*Cell Signaling Technology Cat# D6D9 #RRID:AB_2195767*
Pluripotency marker	*Mouse anti-SSEA4*	*1:100*	*BD Pharmigen Cat# 560073 #RRID:AB_1645601*
Pluripotency marker	*Mouse anti TRA-1-81*	*1:100*	*Milipore Cat# MAB4381 #RRID:AB_177638*
Pluripotency marker	*Mouse anti human SSEA4-PE*	*1:800*	*Stem Cell Technologies Cat# 60062PE.1 #RRID N/A*
Isotype control	*Mouse IgG3 kappa-PE*	*1:6400*	*Stem Cell Technologies Cat# 60073PE.1 #RRID N/A*
Pluripotency marker	*Mouse anti human TRA- 1-60*	*1:400*	*Stem Cell Technologies, Cat #60064PE.1 #RRID N/A*
Isotype control	*PE Mouse IgM*	*1:400*	*BD, Pharmingen Cat# 555584 #RRID:AB_395960*
Ectoderm marker	*Mouse anti-TUJ-1*	*1:500*	*Neuromics Cat# MO15013 #RRID:AB_2737114*
Ectoderm marker	*Rabbit anti-PAX6*	*1:500*	*Covance Cat# PRB-278P #RRID:AB_291612*
Mesoderm marker	*Mouse anti-SMA*	*1:300*	*Sigma Cat #A5228 #RRID:AB_262054*
Mesoderm marker	*Mouse anti-VIMENTIN*	*1:300*	*Abcam Cat# ab8978 #RRID:AB_306907*
Endoderm marker	*Mouse anti-AFP*	*1:20*	*RD Cat# MAB 1368 #RRID:AB_357658*
Endoderm marker	*Goat anti HNF-3β/FOXA2*	*1:100*	*RD Cat# 2400 #RRID:AB_2294104*
Secondary antibody	*Anti-mouse IgG*	*1:500*	*Invitrogen Cat# A11001 #RRID: AB_2534069*
Secondary antibody	*Anti-rabbit*	*1:500*	*Invitrogen Cat# A11002 #RRID:AB_2534070*
Secondary antibody	*Anti-mouse IgM*	*1:500*	*Invitrogen Cat# A21042 #RRID:AB_2535711*
Secondary antibody	*Anti-goat*	*1:500*	*Invitrogen Cat# A11055 #RRID:AB_2534102*

Primers		
	Target	Forward/Reverse primer (5′–3′)
Genotyping	*CERKL (583 bp)*	GAGCAAAACGCTGGTCAGATG/GCTAGTGGGGATGCCAGAAG
Pluripotency marker (RT-PCR)	*OCT4 (165 bp)*	AAGCCCTCATTTCACCAGG CTTGGAAGCTTAGCCAGGTC
Pluripotency marker (RT-PCR)	*SOX2 (181 bp)*	TCACATGTCCCAGCACTACC CCCATTTCCCTCGTTTTTCT
Pluripotency marker (RT-PCR)	*NANOG (260 bp)*	CCAAATTCTCCTGCCAGTGAC CACGTGGTTTCCAAACAAGAAA
Pluripotency marker (RT-PCR)	*REX1 (300 bp)*	CAGATCCTAAACAGCTCGCAGAAT GCGTACGCAAATTAAAGTCCAGA
Pluripotency marker (RT-PCR)	*GDF3 (631 bp)*	CTTATGCTACGTAAAGGAGCTGGG GTGCCAACCCAGGTCCCGGAAGTT
Pluripotency marker(RT-PCR)	*DPPA4 (408 bp)*	GGAGCCGCCTGCCCTGGAAAATTC TTTTTCCTGATATTCTATTCCCAT
House-keeping gene (RT-PCR)	*GAPDH (463 bp)*	ATCGTGGAAGGACTCATGACCACA CCCTGTTGCTGTAGCCAAATTCGT
Sendai virus detection (RT-PCR)	*SeV (181 bp)*	GGATCACTAGGTGATATCGAG C* ACCAGACAAGAGTTTAAGAGATATGTATC*
Sendai virus transgene detection (RT-PCR)	*KOS (528 bp)*	ATGCACCGCTACGACGTGAGCGC ACCTTGACAATCCTGATGTGG
Sendai virus transgene detection (RT-PCR)	*Klf (410 bp)*	TTCCTGCATGCCAGAGGAGCCC AATGTATCGAAGGTGCTCAA*
Sendai virus transgene detection (RT-PCR)	*c-Myc (532 bp)*	TAACTGACTAGCAGGCTTGTCG* TCCACATACAGTCCTGGATGATGATG

Karyotype analysis

The karyotype was analyzed at passage 13 by G-banding at 400–550 band resolution, 30 metaphases analyzed (Service of Biobanco de Sistema Sanitario Público, Granada, Spain).

Fingerprinting

Genomic DNA (gDNA) from 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.

Detection of pluripotency markers and vector elimination by RT-PCR

Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany), 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 of pluripotency markers was analyzed using the primers described in Table 3. Patient's fibroblasts were used as negative control. For Sendai virus elimination test, fibroblasts collected at day 7 after transduction were used as positive control (C+) for Cytotune 2.0 vector detection by RT-PCR. The PCR reaction was performed with MyTaq DNA Polymerase (Bioline GmbH, Luckenwalde, Germany) using Applied Biosystems Veriti Thermal Cycler with following steps: denaturation 94 °C for 15 s, annealing 50–65 °C for 30 s, extension 72 °C for 45 s (x 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

hiPSC colonies cultured in plastic dishes were washed in PBS and

fixed in 4% PFA for 15 min at room temperature (RT). Fixed cells were washed twice in PBS and placed in blocking solution (3% serum, 0.5% Triton-X100 in PBS) for 1 h at RT. Cells were then incubated overnight at 4 °C with primary antibodies diluted in blocking solution (Table 3). The following day, cells were washed three times in PBS and incubated with an appropriate secondary antibody at RT for 1 h. Thereafter, cells were stained with DAPI (1:1000) at RT during 5 min, washed three times in PBS and imaged with a Leica DMi 8 microscope coupled with a DFC/7000GT camera.

Flow cytometry

hiPSCs were dissociated using Accutase (Innovative Cell Technologies) for 2–4 min at RT, centrifuged at 300 rcf for 5 min and 100,000 cells resuspended in 200 µl PBS + 2% FBS. Anti-human SSEA-4 and anti-human TRA-1-60 antibodies, both PE conjugated, were added and incubated for 20 min at RT. Isotype negative controls were used at the same final concentration (Table 3). 7-Aminoactinomycin D (7-AAD, Life Technologies) was used to identify live cells. The cells were analyzed using a CytoFLEX flow cytometer (Beckman Coulter) and data was analyzed by CytExpert 2.0 software. FACS gates were defined according to isotype controls.

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). Ratios of Reading B/ Reading A < 1.0 are considered negative for mycoplasma.

hiPSC nomenclature

The generated hiPSC line was named following Spanish National Stem Cell Bank recommendations. The lines are registered at https://hpscreg.eu/ as RP3-FiPS4F1 (ESi066-A) and Ctrl3-FiPS4F1 (ESi067-A).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101455.

Acknowledgements

We are thankful to the patient and his sibling for generously donating skin biopsies. This work was supported by Fundació La Marató TV3 201417.30 and 201417.32, Instituto de Salud Carlos III (ISCIII) from Spanish Ministry of Science, Innovation and Universities-FEDER (European Regional Development Fund) PI16/00409, Platform for Proteomics, Genotyping and Cell Lines, PRB3 of ISCIII (PT17/0019/0024), SE is supported by Miguel Servet contract, ISCIII-FEDER, CPII16/00037. DL is supported by Miguel Servet contract ISCIII-FEDER, CP18/00033.

Author contributions

DL designed study, performed the experiments, data analyses, manuscript writing. ABA, ML, VBF performed experiments, data analyses, GM, RGD genotyped the patient and coordinated patient sample donation, manuscript writing. SE study design, manuscript writing, financial support.

References

Ávila-Fernandez, A., Riveiro-Alvarez, R., Vallespin, E., Wilke, R., Tapias, I., Cantalapiedra, D., Aguirre-Lamban, J., Gimenez, A., Trujillo-Tiebas, M.J., Ayuso, C., 2008. *CERKL* mutations and associated phenotypes in seven Spanish families with autosomal recessive retinitis pigmentosa. Invest. Ophthalmol. Vis. Sci. 49 (6), 2709–2713. https://doi.org/10.1167/iovs.07-0865.

Tuson, M., Marfany, G., Gonzàlez-Duarte, R., 2004. Mutation of *CERKL*, a novel human ceramide kinase gene, causes autosomal recessive retinitis pigmentosa (RP26). Am. J. Hum. Genet. 74 (1), 128–138.

Verbakel, S.K., van Huet, R.A.C., Boon, C.J.F., den Hollander, A.I., Collin, R.W.J., Klaver, C.C.W., Hoyng, C.B., Roepman, R., Klevering, B.J., 2018. Non-syndromic retinitis pigmentosa. Prog. Retin. Eye Res. 66, 157–186. https://doi.org/10.1016/j.preteyeres.2018.03.005.