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Lab Resource: Multiple Cell Lines

Generation of gene-corrected human induced pluripotent stem cell lines derived from retinitis pigmentosa patient with Ser331Cysfs*5 mutation in MERTK

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A B S T R A C T

The human induced pluripotent stem cell (hiPSC) line RP1-FiPS4F1 generated from the patient with autosomal recessive retinitis pigmentosa (arRP) caused by homozygous Ser331Cysfs*5 mutation in Mer tyrosine kinase receptor (MERTK) was genetically corrected using CRISPR/Cas9 system. Two isogenic hiPSCs lines, with heterozygous and homozygous correction of c.992_993delCA mutation in the *MERTK* gene were generated. These cell lines demonstrate normal karyotype, maintain a pluripotent state, and can differentiate toward three germ layers *in vitro*. These genetically corrected hiPSCs represent accurate controls to study the contribution of the specific genetic change to the disease, and potentially therapeutic material for cell-replacement therapy.

Resource table

Unique stem cell lines identifier	RCPFi002-A RCPFi003-A
Alternative names of stem cell lines	RP1-FiPS4F1-GC1(RCPFi003-A-1) RP1-FiPS4F1-GC2(RCPFi003-A-2)
Institution	Research Center Príncipe Felipe, Eduardo Primo Yufera 3, Valencia, Spain
Contact information of distributor	Slaven Erceg, serceg@cipf.es
Type of cell lines	iPSC
Origin	Human
Cell Source	Dermal fibroblasts
Clonality	Clonal
Method of reprogramming	Sendai virus
Multiline rationale	Gene corrected clones
Gene modification	Yes
Type of modification	Gene correction
Associated disease	Retinitis pigmentosa
Gene/locus	*MERTK* (NM_006343.2) exon 7 (c.992_993delCA)/Locus 2q13
Method of modification	CRISPR
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	*N/A*
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/fd-banco-nacional-lineas-celulares/fd-lineas-celulares-disponibles/lineas-de-celulas-iPS.shtml*
Ethical approval	*Ethics Review Board-competent authority approval obtained by the Valencian Authority for Stem Cell Research (Approval number: S:177–15)*

Resource utility

Retinitis pigmentosa (RP; OMIM 268000) is a genetically

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heterogeneous retinal degeneration with disease progression leading to moderate to severe loss of vision (Hartong et al., 2006). We previously established an *in vitro* model of RP disease by using hiPSC line RP1-FiPS4F1 (registered as RCPFi003-A), derived from patient with a Ser331Cysfs*5 mutation in MERTK diagnosed with an early onset and severe form of autosomal recessive RP (arRP) (Lukovic et al., 2015). The generated corrected hiPSCs, RP1-FiPS4F1-GC1 (heterozygous) and RP1-FiPS4F1-GC2 (homozygous), represent accurate controls to identify the molecular mechanism underlying the specific disease–causing mutation, while removing the contribution of the genetic background to the disease phenotype. These cell lines also represent a potential therapeutic resource for autologous cell-replacement therapy.

Resource details

Isogenic lines with a corrected mutation were generated by CRISPR-enhanced homology directed repair (HDR) using a single stranded oligonucleotide repair template. CRISPR reagents were delivered as ribonucleoprotein complexes consisting of enhanced specificity eSpCas9_1.1. protein (Slaymaker et al., 2016) and chemically synthesised crRNA and tracrRNAs. This provides a highly active, yet short term expression of the CRISPR components, which maximises HDR efficiency, minimises off-target cutting and is less toxic than delivery of dsDNA. After single cell plating, colonies were picked and screened for mutations using high throughput sequencing of individual clones to identify homozygous or heterozygous clones. The strategy employed for correction is outlined in Fig. 1A. Two hiPSC corrected clones were selected and characterized for pluripotency (Tables 1 and 2). DNA sequencing analysis of RP1-FiPS4F1-GC1 and RP1-FiPS4F1-GC2 confirmed the correction of *MERTK* mutation in one (heterozygous correction) and two alleles (homozygous correction), respectively (Fig. 1B, corrected nucleotides marked in red and silent mutation nucleotides marked in blue). The corrected isogenic lines displayed a typical human embryonic stem cell (hESC) colony-like morphology with cells with high nuclear/cytoplasmic ratio and positive alkaline phosphatase activity (Fig. 1C). Pluripotency was assessed by flow cytometry for SSEA-4 and TRA-1-60 pluripotency markers (Fig. 1D) and immunocytochemistry to pluripotency markers OCT4, SOX2, NANOG, SSEA-4 and TRA-1-81 (Fig. 1F). The expression of endogenous pluripotency genes was detected by RT-PCR (Fig. 1E). To test the ability of the hiPSC lines to generate derivatives of three germ layers *in vitro*, the embryoid body (EB) based assay was performed. Spontaneously differentiated cells were immunostained for endodermal marker α- fetoprotein (AFP), ectodermal marker β-III tubulin (TUJ1) and mesodermal marker α-smooth muscle actin (SMA) (Fig. 1G). The genetic fingerprinting was performed and proved their genetic identity to the parental RP1-FiPS4F1 iPSC line and original fibroblasts (available with authors). The hiPSC lines RP1-FiPS4F1-GC1 and RP1-FiPS4F1-GC2 were karyotypically normal (Supplementary Fig. 1A). The presence of mycoplasma was regularly tested and was negative (Supplementary Fig. 1B).

Materials and methods

Cell culture

Human iPSCs (hiPSCs) were cultured in mTesR1 medium (STEMCELL Technologies) on hESC-qualified Matrigel (BD, #354277) coated plates at 37 °C/5% CO_2. Culture medium was replaced daily and passaged using Dispase (STEMCELL Technologies) upon reaching 70–80% confluence (5–7 days) at a 1:10 split ratio.

Gene correction strategy

The c.992_993delCA mutation in the human *MERTK* gene with predictable amino acid change Ser331Cysfs*5 was corrected by a two base insertion (CA) and simultaneous silent mutation of two bases within a neighbouring Ser codon (S328S, AGT > TCT) using CRISPR/Cas9-induced homology directed repair (see Fig. 1A for strategy). The silent mutations were introduced in order to block recutting upon correct HDR to avoid indel formation, and a CRISPR target site was chosen such that it was specific to the alleles lacking the two base insertion (target site: TCATGACAGCCATTACTCAG CGG). CRISPR reagents were introduced by nucleofection (Lonza, P3 buffer, program CA137) of 10^6 cells (passage21) with Cas9-crRNA-tracrRNA ribonucleoprotein (RNP) complexes. Synthetic RNA oligonucleotides (target site: 5′- TCATGACAGCCATTACTCAG-3′, 225 pmol crRNA/tracrRNA, IDT) were annealed by heating to 95 °C for 2 min in duplex buffer (IDT) and cooling slowly, followed by addition of 122 pmol recombinant eSpCas9_1.1 protein (in 10 mM Tris-HCl, pH 7.4, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, PMID: 26628643), and 500 pmol of a 100 nt ssDNA oligonucleotide (5′-TGCTTGATTTGGTACAGATGTGGTAAGGCAGAGG TGTTAAAAATCATGACTGAGC CATTAGACAGCGGATCAGCTTCCTTGA CCTAAACGAAGAGTAAACATGAGGACCGGG -3′, IDT Ultramer) as a homology-directed repair template to introduce the desired deletion and base changes. After recovery, plating at single cell density and colony picking into 96 well plates, clones were screened for heterozygous and homozygous mutations by high throughput sequencing of amplicons spanning the target site using an Illumina MiSeq instrument.

Crude DNA lysates were prepared by incubation of cells in 100 µl of yolk sac lysis buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM MgCl2, 0.45% IGEPAL CA-630, 0.45% Tween 20, 400 g/ml proteinase K) at 60 °C 1 h, 95C 10 min followed by dilution 10× in water. The region surrounding the mutation was amplified by nested PCR using 1 µl diluted lysate and KAPA HiFi hotstart polymerase (Kapa Biosystems) by 35 cycles of (98 °C 20 s, 65 °C 15 s, 72 °C 30 s), using primers MERTK_500_F and MERTK_500_R (see below). Subsequently, reactions were diluted 10× and re-amplified with primers MERTK_miseq_F and MERTK_miseq_R (see below) (Tm = 60 °C) to ensure specificity for the *MERTK* gene. Indexed Illumina sequencing adaptors were added by a third round of PCR to specify the location of positive clones. Final cell lines were further validated by both MiSeq and Sanger sequencing.

Primer sequences (5′-3′).

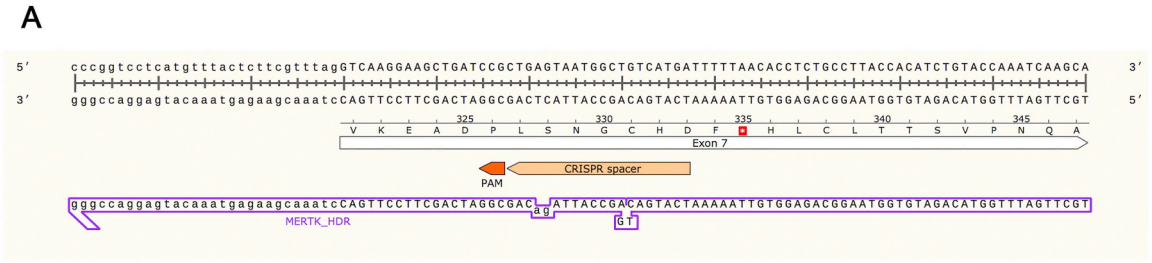
MERTK_500_F: gctccgttacctgccacagtg.
MERTK_500_R: caactctcagtcaggccactg.
MERTK_miseq_F: ACACTCTTTCCCTACACGACGCTCTTCCGATCTc gtagaggaagggccacgtg.
MERTK_miseq_R: TCGGCATTCCTGCTGAACCGCTCTTCCGATCTGG GCTCACTGCAGACCAGC.

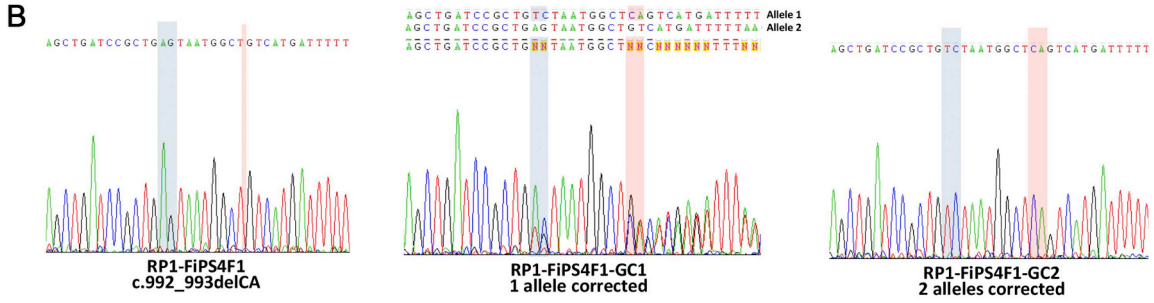
In vitro differentiation assay

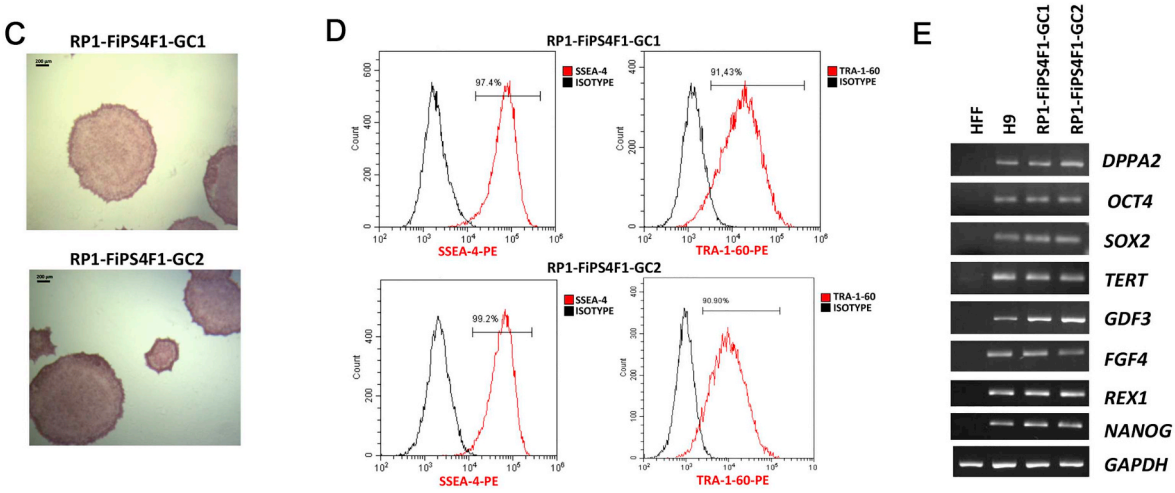
In vitro differentiation was performed by EB formation, namely the hiPSC colonies were lifted manually and cultured in non-adherent conditions in mTeSR1 medium for 24 h, followed by endoderm medium (Knockout DMEM, 10% FBS, 1% Glutamax, NEAA 1%, β-mercaptoethanol 0,23 mM, Penicillin-Streptomycin 1×) for the following 6 days. Thereafter, the EBs were seeded on glass coverslips treated with 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 4% PFA for 15 min and analyzed by immunofluorescence (Table 3). Confocal images were taken by Leica SP8 microscope.

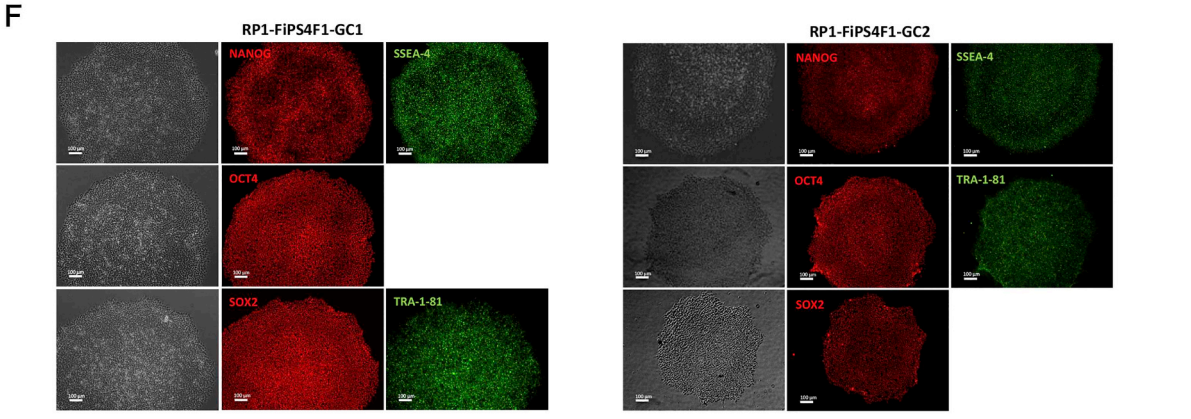
Karyotype analysis

The karyotype was analyzed at passage 35 (7 passages after gene correction) by G-banding at 400–550 band resolution, 30 metaphases analyzed (Service of Biobanco de Sistema Sanitario Público, Granada,









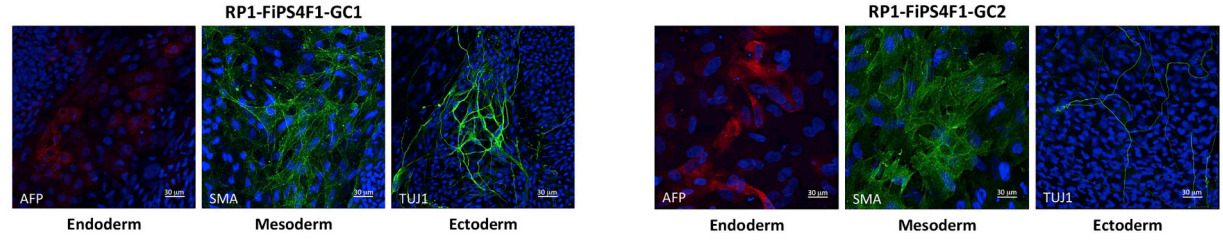


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Fig. 1. Characterization of RP1-FiPS4F1-GC1 and RP1-FiPS4F1-GC2 cell lines. A. Gene correction strategy. DNA sequence upstream and downstream of *MERTK* c.992_993delCA with the predictable stop codon at position 335. crRNA sequence and ssDNA oligonucleotide template showing the position of inserted nucleotides (GT) and the silent mutation (ag). B. DNA electropherograms showing the *MERTK* c.992_993delCA mutation in the original cell line (RP1-FiPS4F1) and the sequence in the same region of corrected, RP1-FiPS4F1-GC1 and RP1-FiPS4F1-GC2, cell lines. Silent mutation nucleotides are marked by a blue stripe. C. Alkaline phosphatase staining of RP1-FiPS4F1-GC1 and RP1-FiPS4F1-GC2 colonies. D. Flow cytometry of surface pluripotency markers SSEA-4 and TRA-1-60. E. RT-PCR analysis of pluripotency markers. F. Immunocytochemistry for pluripotency markers OCT4, SOX2, NANOG, SSEA-4, TRA-1-81. Nuclei were counterstained with DAPI (blue). G. Immunocytochemistry for ectodermal (TUJ1), endodermal (AFP) and mesodermal (SMA) markers. Nuclei were stained with DAPI (blue).

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
RP1-FiPS4F1-GC1 (RCPFi003-A-1)	*N/A*	Male	45	Caucasian	*MERTK* (NM_006343.2), heterozygous c.[992_993delCA]	Retinitis pigmentosa (RP)(corrected)
RP1-FiPS4F1-GC2 (RCPFi003-A-2)	*N/A*	Male	45	Caucasian	MERTK, (NM_006343.2), WT	Retinitis pigmentosa (RP)(corrected)

Spain).

Fingerprinting

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

Mutation sequencing

Genomic DNA from hiPSCs was isolated using the QIAamp DNA Blood mini kit (Qiagen, Hilden, Germany). Primers used for amplification and directed sequencing of *MERTK* upstream and downstream of c.992_993delCA were as follows: 5´CGAAGAGGTTCTAAGAGAGG3´ and 5´CCATTTTCATCAGTCGCCTC3´ (annealing temperature 55 °C).

Detection of pluripotency markers 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, Hilden, Germany) was used to carry out cDNA synthesis from 1 μg of total RNA according to the manufacturer's instructions. The PCR reaction was performed with MyTaq DNA Polymerase (Bioline GmbH, Germany) using Applied Biosystems Veriti Thermal Cycler. The expression level of pluripotency markers was analyzed using the primers described in Table 3. Human foreskin fibroblasts (HFF) and hESC H9 (WiCell) were used as negative and positive control, respectively.

Immunocytochemistry

Cells 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. 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 visualized on Leica DMi 8 microscope coupled with DFC/7000GT camera and Leica SP8 confocal microscope. Samples grown on coverslips were mounted using Vectashield.

Flow cytometry

hiPSCs were dissociated using Accutase (Innovative Cell Technologies) for 2–4 min at RT, centrifuged at 300 rcf for 5 min and resuspended in PBS + 2% FBS. Anti-human SSEA-4 and Anti-human TRA-1-60 antibodies were added and incubated for 20 min at RT. IgG3, kappa isotype and IgM, kappa isotype were used as negative control of SSEA-4 and TRA-1-60, respectively. The cells were analyzed using a CytoFLEX flow cytometer (Beckman Coulter) and data analyzed by CytExpert 2.0 software.

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	*Normal*	*Shown by immunocytochemistry*
Phenotype	Qualitative analysis *(Immunocytochemistry)*	*Positive staining/expression of pluripotency markers: Oct4, Nanog, Sox2, SSEA4, TRA-1-81*	Fig. 1*panel F*
	Quantitative analysis *(Flow cytometry)*	*Assess % of positive cell surface markers TRA- 1-60: 91,43%/ 90,90% SSEA-4: 97,4%/99,2%*	Fig. 1*panel D*
Genotype	Karyotype (G-banding) and resolution	*46XY, Resolution 450–500*	*Supplementary Fig. 1A*
Identity	Microsatellite PCR (mPCR)	N/A	N/A
	STR analysis	*10 loci analyzed, all matching*	*Available with authors*
Mutation analysis (IF APPLICABLE)	Sequencing	*MERTK* (NM_006343.2), c.992_993delCA	Fig. 1*panel B*
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	*Mycoplasma testing by luminescence. Negative*	*Supplementary Fig. 1B*
Differentiation potential	Embryoid body formation	*Positive TUJ1 ectodermal staining, positive SMA mesodermal staining and positive AFP 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

Table 3
Reagents 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*
Pluripotency marker	*Rabbit anti-Oct4*	*1:400*	*Cell Signaling Technology Cat# C30A3*
Pluripotency marker	*Rabbit anti-Sox2*	*1:400*	*Cell Signaling Technology Cat# D6D9*
Pluripotency marker	*Mouse anti-SSEA4*	*1:100*	*BD Pharmigen Cat# 560073*
Pluripotency marker	*Mouse IgM anti-TRA-1-81*	*1:100*	*Millipore Cat# MAB4381*
Pluripotency marker	*Mouse anti-Human SSEA-4-PE*	*1:100*	*STEMCELL Technologies Cat# 60062PE.1*
Pluripotency marker	*Mouse anti-Human TRA-1-60*	*1:400*	*STEMCELL Technologies Cat# 60064PE.1*
Differentiation marker	*Mouse anti-BTubulin (TUJ1)*	*1:500*	*Neuromics Cat# MO15013*
Differentiation marker	*Mouse anti-SMA*	*1:300*	*Sigma Cat# A5228*
Differentiation marker	*Rabbit anti-α-fetoprotein (AFP)*	*1:20*	*Dako Cat# A0008*
Secondary antibody	*Goat anti-mouse IgG*	*1:500*	*Invitrogen Cat# A11001*
Secondary antibody	*Goat anti-rabbit IgG*	*1:500*	*Invitrogen Cat# A11012*
Secondary antibody	*Goat anti-mouse IgM*	*1:500*	*Invitrogen Cat# A21042*
Isotype control	*Mouse IgG3 kappa-PE*	*1:800*	*STEMCELL Technologies Cat# 60073PE.1*
Isotype control	*Mouse IgM kappa-PE*	*1:40*	*BD Pharmigen Cat# 555584*

Primers	*Target*	Forward/Reverse primer (5′-3′)
Pluripotency marker (RT-PCR)	*OCT4*	AAGCCCTCATTTCACCAGG CTTGGAAGCTTAGCCAGGTC
Pluripotency marker (RT-PCR)	*NANOG*	CCAAATTCTCCTGCCAGTGAC CACGTGGTTTCCAAACAAGAAA
Pluripotency marker (RT-PCR)	*SOX2*	TCACATGTCCCAGCACTACC CCCATTTCCCTCGTTTTTCT
Pluripotency marker (RT-PCR)	*TERT*	TGGCTGCGTGGTGAACTTG GCGGTTGAAGGTGAGACTGG
Pluripotency marker (RT-PCR)	*FGF4*	CTACAACGCCTACGAGTCCTACA GTTGCACCAGAAAAGTCAGAGTTG
Pluripotency marker (RT-PCR)	*REX1*	CAGATCCTAAACAGCTCGCAGAAT GCGTACGCAAATTAAAGTCCAGA
Pluripotency marker (RT-PCR)	*GDF3*	CTTATGCTACGTAAAGGAGCTGGG GTGCCAACCCAGGTCCCGGAAGTT
Pluripotency marker (RT-PCR)	*DPPA2*	CCGTCCCCGCAATCTCCTTCCATC ATGATGCCAACATGGCTCCCGGTG
House-keeping gene (RT-PCR)	*GAPDH*	ATCGTGGAAGGACTCATGACCACA CCCTGTTGCTGTAGCCAAATTCGT

Alkaline phosphatase staining

Alkaline phosphatase staining was carried out using Alkaline Phosphatase Staining Kit II (Stemgent, Cambridge, MA, USA) according to manufacturer's instructions.

Mycoplasma detection

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

hiPSC nomenclature

The generated hiPSC lines were named following Spanish National Stem Cell Bank recommendations. The lines are registered at https://hpscreg.eu/as RCPFi003-A-1 (RP1-FiPS4F1-GC1) and RCPFi003-A-2 (RP1-FiPS4F1-GC2) lines.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.11.003.

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References

Hartong, D.T., Berson, E.L., Dryja, T.P., 2006. Retinitis pigmentosa. Lancet 368, 1795–1809.

Lukovic, D., Artero Castro, A., Delgado, A.B., Bernal Mde, L., Luna Pelaez, N., Diez Lloret, A., Perez Espejo, R., Kamenarova, K., Fernandez Sanchez, L., Cuenca, N., Corton, M., Avila Fernandez, A., Sorkio, A., Skottman, H., Ayuso, C., Erceg, S., Bhattacharya, S.S., 2015. Human iPSC derived disease model of MERTK-associated retinitis pigmentosa. Sci. Rep. 5, 12910.

Slaymaker, I.M., Gao, L., Zetsche, B., Scott, D.A., Yan, W.X., Zhang, F., 2016. Rationally engineered Cas9 nucleases with improved specificity. Science 351, 84–88.