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Lab Resource: Stem Cell Line

Generation of an iPSC line, INMi001-A, carrying the two most common *USH2A* mutations from a compound heterozygote with non-syndromic retinitis pigmentosa

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A B S T R A C T

We generated an induced pluripotent stem cell (iPSC) line from a patient with non-syndromic retinitis pigmentosa who is a compound heterozygote for the two most frequent *USH2A* variants, c.2276G > T and c.2299delG localized in exon 13. Patient fibroblasts were reprogrammed using the non-integrative Sendai virus reprogramming method and the human OSKM transcription factor cocktail. The generated cells were pluripotent and genetically stable. This iPSC line will be an important tool for studying the pathogenesis of these *USH2A* mutations and for developing treatments that, due their high prevalence, will target a large patient population.

Resource table

Unique stem cell line identifier	INMi001-A
Alternative name(s) of stem cell line	*USH2A*-RP-iPSC
Institution	Institute for Neurosciences of Montpellier, Montpellier, France
Contact information of distributor	Vasiliki Kalatzis vasiliki.kalatzis@inserm.fr
Type of cell line	iPSC
Origin	Human
Additional origin info	Age: 70 years old Sex: Female Ethnicity: Caucasian
Cell Source	Dermal fibroblasts
Clonality	Clonal
Method of reprogramming	Non-integrative Sendai virus vectors
Genetic Modification	Yes
Type of Modification	Congenital mutation
Associated disease	Non-syndromic retinitis pigmentosa
Gene/locus	*USH2A*, 1q41
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	March 2018
Cell line repository/bank	https://hpscreg.eu/cell-line/INMi001-A
Ethical approval	Regional committee: CPP Southern Mediterranean I (2014-A00549–38) National committee: ANSM (140549B-62)

Resource utility

This iPSC line carries two recurrent *USH2A* variants, the hypomorphic c.2276G > T and the deleterious c.2299delG, and is associated with autosomal recessive retinitis pigmentosa (RP). It will prove an invaluable tool for deciphering the pathophysiology of non-syndromic and syndromic RP, both caused by mutations in *USH2A*.

Resource details

Retinitis pigmentosa (RP) is an inherited retinal dystrophy (IRD) characterized by progressive retinal degeneration, which results in night blindness and subsequently peripheral vision loss. Mutations in over 80 genes have been described to cause RP (Verbakel et al., 2018). Interestingly, mutations in *USH2A* can either cause Usher syndrome type 2 (USH2), presenting with RP and hearing loss, or isolated RP, in which only the retina is affected (Kremer et al., 2006). The different phenotypes found in patients with mutations in *USH2A*, was suggested to be due to an allelic hierarchy of *USH2A* mutations. Retina-specific alleles in *USH2A*, are considered to segregate in patients presenting non-syndromic RP and normal hearing. The recurrent missense variant c.2276G > T; p.Cys759Phe is one of these retina-specific alleles (Lenassi et al., 2015). In the present study, we have generated an iPSC line from a patient presenting autosomal recessive RP carrying this

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allele. Furthermore, this individual is compound heterozygous for another recurrent *USH2A* variant, c.2299delG; p.Glu767Serfs*21, which, in the homozygous state, is associated with USH2.

The human iPSC line, INMi001-A (*USH2A*-RP-iPSC), has been generated using a non-integrative method, the CytoTune™-iPS 2.0 Sendai Reprogramming Kit. Human dermal fibroblasts were obtained from a patient skin biopsy and reprogrammed using the Sendai virus vectors containing the reprogramming factors *OCT3/4, SOX2, KLF4* and *c-MYC,* which confer pluripotency to somatic cells (Takahashi et al., 2007). After reprogramming, *USH2A*-RP-iPSC colonies displayed the typical morphology of tightly packed cells surrounded by a distinct border (Fig. 1A). We assessed the loss of the Sendai vectors over successive passages (tested at P8 and P18) by reverse transcription (RT)-PCR using specific primers for each transgene (Fig. 1B). By comparison, as a negative control, non-transduced patient fibroblasts (Fibro) did not carry the Sendai vectors whereas, as a positive control, transduced fibroblasts (Fibro + SeV) expressed all three vectors. The genetic integrity of the *USH2A*-RP-iPSC line was determined by karyotype analysis performed after 12 feeder-free passages, which showed a normal karyotype (46, XX), ruling out major chromosome abnormalities as a result of the reprogramming process (Fig. 1C). The pluripotency of the generated iPSC line was evaluated by the endogenous expression of the pluripotency-associated transcription factors *NANOG*, *OCT3/4* and *LIN28a* by quantitative real time PCR (q-PCR). As shown in Fig. 1D, *USH2A*-RP-iPSC expressed these genes in contrast to the fibroblast controls (Fibro; Fibro + SeV), indicating the pluripotent state. This was further confirmed by immunofluorescence staining of antibodies specific to NANOG, OCT3/4 and SOX2 (Fig. 1E). The ability of the *USH2A*-RP-iPSC line to differentiate into the three germ layers was explored *in vitro* using an embryoid body (EB) assay. The corresponding EBs expressed markers of the three germ layers as assessed by immunofluorescence staining of Glial Fibrillary Acidic Protein (GFAP) for ectoderm, Smooth Muscle Actin (SMA) for mesoderm and α-Fetoprotein (AFP) for endoderm (Fig. 1E). We verified the presence of the two *USH2A* variants c.2276G > T and c.2299delG, segregating with the retinal phenotype in the patient, in the *USH2A*-RP-iPSC line by Sanger sequencing, in comparison to a wild type control (Fig. 1F). Furthermore, the identity of the patient iPSC line was confirmed by microsatellite PCR analysis in comparison to fibroblasts of the same individual and wild type iPSC (Suppl. File 1). Lastly, the generated *USH2A*-RP-iPSC line was confirmed to be free of mycoplasma contamination (Suppl. File 2).

Materials and methods

Human dermal fibroblast cell culture

Dermal fibroblast derived from a skin biopsy were cultured in AmnioMAX C100 basal media with GlutaMAX (Gibco) containing 10% decomplemented foetal calf serum (FCS; Lonza), 1% penicillin-streptomycin-amphotericin B (Lonza) and 2% AmnioMax-C100 supplement (Gibco).

Reprogramming of USH2A-RP-iPSC fibroblasts

Fibroblasts were reprogrammed using the integration-free CytoTune™-iPS 2.0 Sendai Reprogramming Kit. Fibroblasts were seeded in high glucose DMEM containing GlutaMAX (Gibco) and supplemented with 10% FCS (Lonza), 1% non-essential amino acids (NEAA) (Gibco) and 55 mM β-mercaptoethanol (Gibco). Cells were transduced according to the manufacturer's instructions. The medium was refreshed daily for 7 days before the cells were passaged onto plates coated with Matrigel hESC-Qualified Matrix (Corning). On day 8, the medium was changed to TeSR-E7 Basal Medium (Stemcell Technologies) and was refreshed daily until iPSC colonies emerged. The colonies were mechanically passaged and cultured in Essential 8 (E8) medium (Gibco). Passages were subsequently performed using Versene solution (Gibco).

Karyotype analysis

iPSC were grown to 50% confluence and incubated for 3 h in E8 containing 0.1 μg/ml Nocodazole (Sigma-Aldrich). Cells were dissociated using TrypLE (Gibco), pelleted (200g, 10 min, RT), gently resuspended in buffered hypotonic solution (Genial Genetics) and incubated 20 min at 37 °C. The reaction was stopped with ice-cold fixation solution (3 vol. methanol: 1 vol. glacial acetic acid), prior to centrifugation at 200*g* for 10 min at 4 °C. The pellet was washed, resuspended in ice-cold fixation solution and stored at −20 °C until analysis. Twenty metaphase spreads were counted and analyses were performed by the Chromostem facility (CHU Montpellier, France).

RT-PCR and qPCR analysis

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN) and subjected to first strand cDNA synthesis using the SuperScript® III First-Strand Synthesis System (Life Technologies) and random hexamers (Life Technologies) according to the manufacturer's protocols. RT-PCR was performed as follows: denaturation (95 °C, 10 min), amplification (95 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min for 35 cycles), and a final extension (72 °C, 10 min). For qPCR analysis, the cDNA was diluted to 2.5 ng/μl and 2 μl was used for each reaction with the FastStart SYBR Green I Master mix on a LightCycler 480 II thermal cycler (Roche). Gene expression was normalized to *GAPDH* expression. All primers used in this study are listed in Table 2.

Immunofluorescence staining

Cells were fixed using 4% PFA and permeabilized using 0.1% Triton X-100 (Sigma-Aldrich). Non-specific binding was blocked with 1% BSA and 10% donkey serum (Millipore). Primary antibodies were incubated overnight at 4 °C (as a negative control, no primary antibody was used) and fluorescence-conjugated secondary antibodies 1 h at RT (Jackson ImmunoResearch). Nuclei were stained with 0.2 μg/ml bisBenzimide (Sigma-Aldrich). Cells were observed using a Zeiss ApoTome 2 Upright wide-field microscope.

In vitro differentiation assay

iPSC were dissociated with Accutase (Stemcell Technologies) and seeded on ultra-low attachment dishes for 2 days in E8 containing Y27632 StemMACS. At day 3, the medium was changed to DMEM/F12 (Gibco) supplemented with 20% Knockout serum replacement (Gibco), 1% penicillin-streptomycin (Gibco), 1% GlutaMax, 55 mM β-mercaptoethanol and 1% NEAA. At day 7, the embryoid bodies were seeded onto Matrigel-coated wells and culture for a further 10 days before immunofluorescence staining.

Mutation analysis

Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen) and PCR-amplified using *USH2A*-specific primers (Table 2). The dNTPs were removed using the ExoSAP-IT PCR Clean-up kit (GE Healthcare) and the amplicon sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit V3.1 on an Applied Biosystems 3130xL Genetic Analyzer.

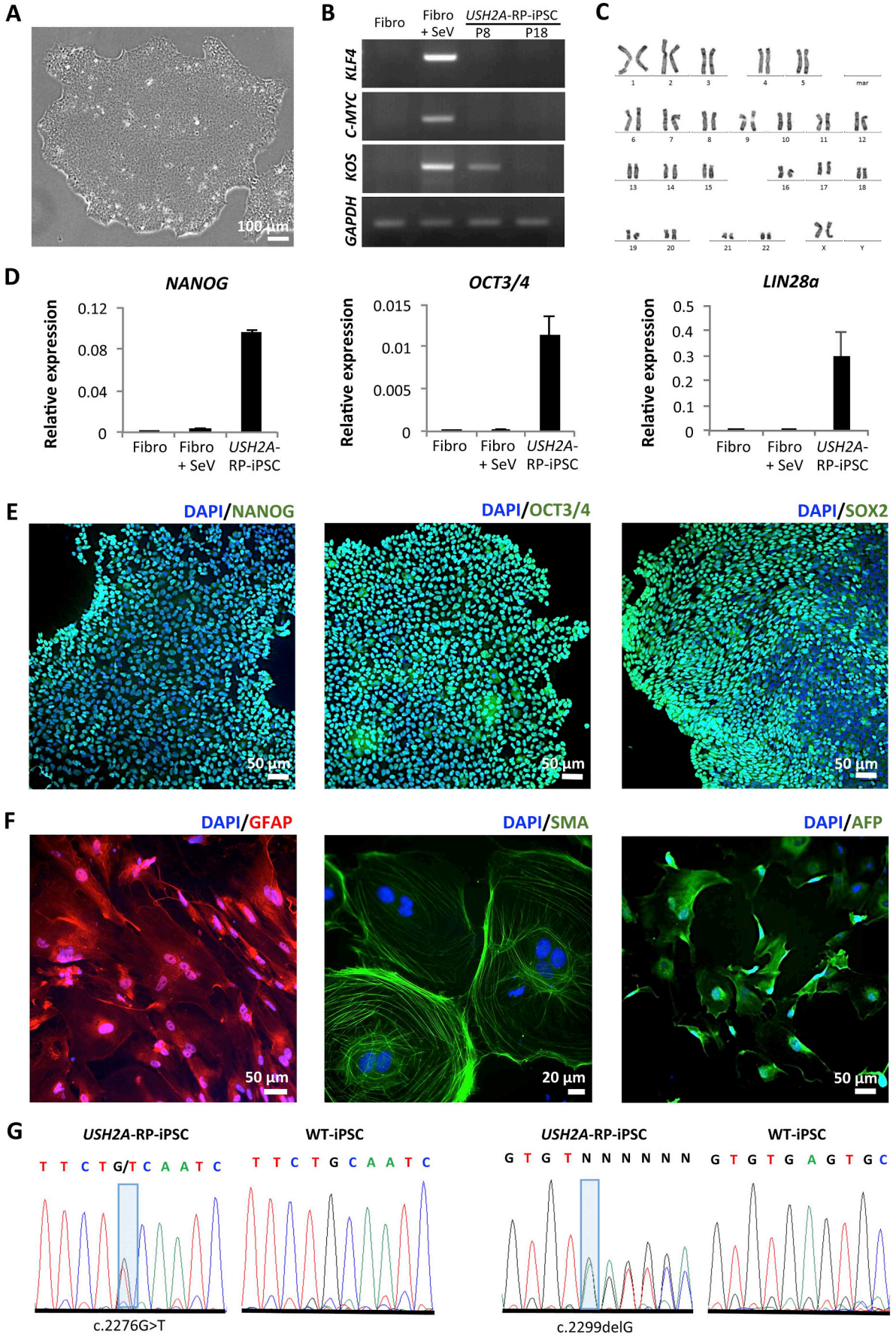

Fig. 1. Characterization of the INMi001-A (*USH2A*-RP-iPSC) line

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Microscopy	Normal morphology	Fig. 1A
Phenotype	Qualitative analysis Immunofluorescence staining	Positive for pluripotency markers: NANOG, OCT3/4 and SOX2	Fig. 1E
	Quantitative analysis qPCR	Expression of pluripotency markers: *NANOG*, *OCT3/4*, *LIN28a*	Fig. 1D
Genotype	Karyotype (G-banding) and resolution	46XX, Resolution 400–500	Fig. 1C
Identity	Microsatellite PCR (mPCR) OR	DNA Profiling Performed D3S306 D8S532 D11S4191 D17S787 D19S572	Suppl. file 1
	STR analysis	Not performed	N/A
Mutation analysis	Sequencing	Compound heterozygous, *USH2A* c.2276G > T and c.2299delG	Fig. 1G
	Southern Blot OR WGS	Not performed	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing luminescence. Negative	Suppl. file 2
Differentiation potential	Embryoid body formation	Expression of GFAP (ectoderm), SMA (mesoderm) and AFP (endoderm) in iPSC-derived EBs	Fig. 1F
Donor screening (OPTIONAL)	Hepatitis A, Hepatitis B, Hepatitis C, HIV 1–2	Negative	Not shown but available with authors
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry

	Antibody	Dilution	Company cat # and RRID
Pluripotency Markers	Rabbit anti-SOX2	1/200	Thermo Fisher Scientific Cat# 48–1400, RRID:AB_2533841
Pluripotency Markers	Rabbit anti-NANOG	1/200	Abcam Cat# ab21624, RRID:AB_446437
Pluripotency Markers	Mouse anti-OCT3/4	1/200	Santa Cruz Biotechnology Cat# sc-5279, RRID:AB_628051
Differentiation Markers	Rabbit anti-GFAP	1/200	Dako Cat# Z0334, RRID:AB_10013382
Differentiation Markers	Mouse anti-SMA	1/200	Dako Cat# M0851, RRID:AB_2223500
Differentiation Markers	Mouse anti-AFP	1/200	Sigma Aldrich Cat# WH0000174M1, RRID:AB_1839587
Secondary antibodies	Alexa Fluor® 488 AffiniPure Donkey Anti-Rabbit IgG (H + L)	1/500	Jackson ImmunoResearch, Cat# 711–545-152, RRID: AB_2313584
Secondary antibodies	Alexa Fluor® 488 AffiniPure Donkey Anti-Mouse IgG (H + L)	1/500	Jackson ImmunoResearch, Cat# 715–545-150, RRID: AB_2340846
Secondary antibodies	Alexa Fluor® 647 AffiniPure Donkey Anti-Rabbit IgG (H + L)	1/500	Jackson ImmunoResearch, Cat# 711–606-152, RRID: AB_2340625

Primers

	Target	Forward/Reverse primer (5′-3′)	Product size (bp)
Exogenous reprogramming transgene (RT-PCR)	*KOS*	ATGCACCGCTACGACGTGAGCGC ACCTTGACAATCCTGATGTGG	528
Exogenous reprogramming transgene (RT-PCR)	*KLF4*	TTCCTGCATGCCAGAGGAGCCC AATGTATCGAAGGTGCTCAA	410
Exogenous reprogramming transgene (RT-PCR)	*c-MYC*	TAACTGACTAGCAGGCTTGTCG TCCACATACAGTCCTGGATGATGATG	532
Pluripotency marker (RT-qPCR)	*NANOG*	CAAAGGCAAACAACCCACTT TCTGCTGGAGGCTGAGGTAT	158
Pluripotency marker (RT-qPCR)	*OCT3/4*	GTACTCCTCGGTCCCTTTCC CAAAAACCCTGGCACAAACT	168
Pluripotency marker (RT-qPCR)	*LIN28a*	GGGGAATCACCCTACAACCT CTTGGCTCCATGAATCTGGT	166
GAPDH (RT-qPCR and RT-PCR)	*GAPDH*	AACCATGAGAAGTATGACAAC CTTCCACGATACCAAAGTT	112
USH2A exon 13	*USH2A*	GAAGTTCATCGCAAACAGTTG CACTGATTACAGCGAAGACCTG	686

Microsatellite PCR analysis

Genomic DNA was amplified using primers for informative markers (Table 1). The PCR products were mixed with Genescan 400HD ROX size standard and subsequently analyzed on an Applied Biosystems 3130xL genetic analyzer.

Mycoplasma analysis

Mycoplasma detection was performed on cell culture supernatant using the MycoAlert Mycoplasma Detection Kit (Lonza), according to the manufacturer's instructions, and a CLARIOstar microplate reader (BMG Labtech).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.11.004.

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