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Lab Resource: Multiple Cell Lines

Generation of two induced pluripotent stem cell lines from a patient with compound heterozygous mutations in the *USH2A* gene

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A B S T R A C T

The human iPSC lines LEIi010-A and LEIi010-B were generated from the dermal fibroblasts of a patient with Usher syndrome using episomal plasmids containing *OCT4*, *SOX2*, *KLF4*, *L-MYC*, *LIN28*, mir302/367 microRNA and shRNA for *p53*. These iPSC lines carry compound heterozygous mutations (c.949C > A and c.1256G > T) in *USH2A*. LEIi010-A and LEIi010-B expressed pluripotent stem cell markers, had a normal karyotype and could be differentiated into endoderm, mesoderm and ectodermal lineages.

Resource table

Unique stem cell lines identifier	LEIi010-A LEIi010-B
Alternative names of stem cell lines	1011ips1 1011ips2
Institution	Lions Eye Institute
Contact information of distributor	Dr Fred Chen, fredchen@lei.org.au Dr. Samuel McLenachan, smclenachan@lei.org.au
Type of cell lines	iPSC
Origin	Human
Cell Source	Dermal fibroblasts
Clonality	Clonal
Method of reprogramming	Episomal plasmids
Multiline rationale	Isogenic clones
Gene modification	NO
Type of modification	N/A
Associated disease	Usher Syndrome
Gene/locus	1q41, USH2A
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	9 September 2016
Cell line repository/bank	N/A
Ethical approval	Sir Charles Gairdner Hospital Human Research Ethics Committee (2001–053); Human Research Ethics Office, University of Western Australia (RA/4/1/7916)

Resource utility

Usher syndrome is the most common cause of deafness-blindness. We report the generation of two iPSC lines from a patient with compound heterozygous mutations in *USH2A*. These fully characterized iPSC lines represent a valuable resource for research into Usher syndrome.

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Resource details

Usher syndrome (USH) is an autosomal recessive disease characterized by hearing loss and progressive visual dysfunction in the form of retinitis pigmentosa (RP). With a prevalence of 3.2–6.2/100,000, USH causes approximately 5% of congenital deafness and 18% of RP cases worldwide (Millan et al., 2011; Toms et al., 2015). USH is classified into 3 clinical subtypes (USH1–3) caused by mutations in at least 10 genes, with *USH2A* mutations representing the most common cause (Millan et al., 2011). Here, we report the generation and characterization of two iPSC lines from a patient with the most prevalent subtype, USH2, caused by compound heterozygous mutations in *USH2A*. This female patient had congenital mild neurosensory hearing loss and presented with asymptomatic peripheral retinal pigmentation with optic nerve head drusen at the age of 14 years. Despite having 10° of residual visual field remaining, she was unaware of any vision loss until development of macular oedema at age 19 years. Full-field electroretinography showed flat dark-adapted and light-adapted responses consistent with RP. Genetic analysis using a retinal dystrophy next generation sequencing (NGS) panel identified two previously reported substitution mutations, in exon 6 (c.949C > A p.(=, Tyr318Cysfs*17)) (Vache et al., 2010) and exon 7 (c.1256G > T p.(Cys419Phe)) (Weston et al., 2000) of the *USH2A* gene (NM_206933.2). Segregation analysis demonstrated the mutations were biallelic.

Dermal fibroblasts were cultured from a patient skin biopsy sample and reprogrammed using episomal plasmids expressing *OCT4*, *SOX2*, *KLF4*, *L-MYC*, *LIN28*, *p53*shRNA, and the miR-302/367 cluster. Two clonal iPSC lines (LEIi010-A and LEIi010-B) were established for further characterization (Fig. 1, Tables 1–2). Both LEIi010-A and LEIi010-B displayed morphology typical of pluripotent stem cell colonies (Fig. 1A, scale bars indicate 1 mm) and carried the two *USH2A* variants detected in the patient (Fig. 1A, chromatograms). Digital karyotyping using the Illumina HumanCoreExome-24 Beadchip assay (D'Antonio et al., 2017) with genome-wide copy number variation (CNV) profiling demonstrated both lines were female and had a normal diploid karyotype (46, XX). Analysis of the B allele frequencies (right panels) and LogR ratios (left panels) of 500,000 single nucleotide polymorphisms located across the genome demonstrated an absence of chromosomal rearrangements in LEIi010-A and LEIi010-B (Fig. 1B). The expression of the pluripotency markers (OCT4, NANOG, SOX2 and SSEA4) was demonstrated by immunostaining (Fig. 1C, scale bars indicate 100 μm). Quantitative reverse transcription polymerase chain reaction (RT-qPCR) demonstrated *OCT4*, *NANOG*, *SOX2* and *KLF4* mRNA was expressed at similar levels in LEIi010-A and LEIi010-B iPSC, as well as a control human iPSC line (HuiPSC, Fig. 1D). The capacity of LEIi010-A and LEIi010-B to differentiate into three germ layers was confirmed by the expression of markers of ectoderm (*PAX6, DCX*), mesoderm (*TBXT*) and endoderm (*AFP, SOX7*) lineages in differentiating embryoid body (EB) cultures by RT-qPCR. After 2 weeks of spontaneous differentiation, EBs derived from LEIi010-A and LEIi010-B increased expression of ectoderm, mesoderm and endoderm markers, compared with undifferentiated iPSC (Fig. 1E). Analysis of 16 microsatellite markers confirmed both LEIi010-A and LEIi010-B were derived from the patient's fibroblasts (Fig. 1F). PCR testing demonstrated both LEIi010-A and LEIi010-B were negative for mycoplasma (259 bp band, Fig. 1G). Reprogramming episomes could be detected by PCR in DNA isolated from transfected human fibroblasts, (FB, 95 bp band) but were not detected in LEIi010-A and LEIi010-B iPSC at passage 12 and 13 (p12, p13), respectively (Fig. 1H).

Materials and methods

Cell culture and reprogramming

Patient fibroblasts were cultured in DMEM medium supplemented with 10% fetal calf serum and antibiotic-antimycotic (ThermoFisher). Patient iPSCs were cultured in feeder-free conditions, on geltrex-coated (ThermoFisher) culture plates in TeSR-E8 medium (StemCell Technologies). Patient fibroblasts were reprogrammed using the Episomal iPSC Reprogramming Plasmid kit (SC900A-1, System Biosciences), according to the manufacturer's instructions. Colonies were picked for clonal expansion on day 25. The c.949C > A and c.1256G > T mutations were confirmed in patient iPSCs by PCR amplification followed by Sanger sequencing Primer sequences are listed in Table 3. A commercial iPSC line was cultured in parallel with patient iPSC lines (ThermoFisher, Cat#A18945).

iPSC differentiation

For trilineage differentiation, iPSCs were cultured as spontaneously differentiating EBs. IPSCs were treated with Gentle Cell Dissociation buffer (StemCell Technologies) for 5 min at room temperature, the buffer was then removed and replaced with TeSR-E8 media containing 10 μM Y27632 (Abcam) without disturbing colonies. Colonies were mechanically scraped into the media, and then seeded into ultra-low attachment cell culture plates in TeSR-E8 media supplemented with 10 μM Y27632. On day 3, the media was changed to DMEM/F12 supplemented with 20% knockout serum replacement and antibiotic-antimycotic (ThermoFisher). Media was changed every second day. On day 14, RNA was harvested from EBs for RT-qPCR analysis. Trilineage differentiation was assessed by the induction of genes for ectoderm (*PAX6, DCX*), mesoderm *(TBXT*) and endoderm (*AFP*, *SOX7*).

RT-qPCR

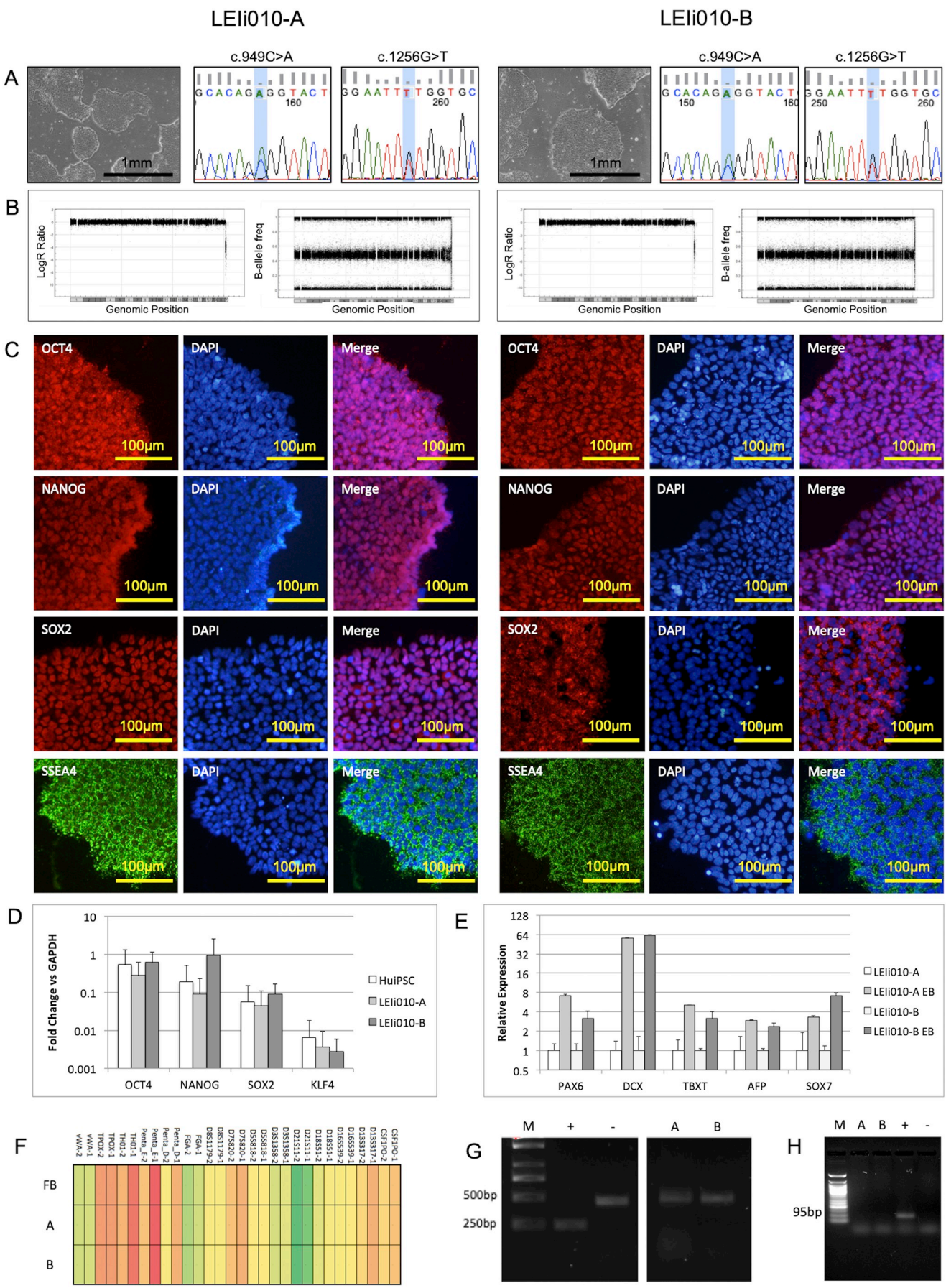
Total mRNA was isolated using TRIZOL and cDNA was synthesized using the RT2 First Strand Kit (Qiagen). qPCR was performed using the CFX Connect Real-Time System (BioRad). Gene expression was quantified using the ΔΔCT method, normalized to *GAPDH* expression. Primers used are listed in Table 3.

Immunostaining

Cells were fixed with 4% paraformaldehyde, washed, then permeabilized using phosphate buffered saline (PBS) with 0.1% Triton X-100 for 15 min. The cells were then incubated in 5% BSA in PBS for 1 h at room temperature. Primary antibodies were applied at 4 °C overnight. Secondary antibodies were applied for 2 h at room temperature. Nuclei were stained with DAPI. Antibodies used are listed in Table 3.

Digital karyotyping

Digital karyotyping was performed on LEIi010-A and LEIi010-B iPSC using the Illumina HumanCoreExome-24 Beadchip SNP array (D'Antonio et al., 2017). CNV analysis was performed on GenomeStudio 2.0 software using the CNVpartition 2.0 plugin (Illumina). B-allele frequencies and LogR ratios were plotted against genomic location.



(caption on next page)

Fig. 1. LEIi010-A (left side) and LEIi010-B (right side) displayed morphologies typical of pluripotent stem cell colonies (images). Sanger sequencing of the USH2A gene demonstrated the presence of the c.1205C > A mutation in the parental fibroblasts used to generate the LEIi009-A and LEIi009-A iPSC lines (chromatograms). **B**: Digital karyotyping of LEIi010-A (left panel) and LEIi010-B (right panel) was performed using the Illumina HumanCoreExome-24 Beadchip SNP array. B-allele frequencies and LogR ratios were plotted against genomic location for 500,000 human SNPs, demonstrating normal 46,XX karyotypes in both lines. **C**: The expression of pluripotency markers (OCT4, NANOG, SOX2 and SSEA-4) was demonstrated in LEIi010-A (left panels) and LEIi010-B (right panels) by immunocytochemistry. Nuclei were counterstained with DAPI. **D**: Quantitative RT-PCR analysis demonstrated similar levels of expression of pluripotency genes (OCT4, NANOG, SOX2 and KLF4) in LEIi010-A and LEIi010-B and a commercial human iPSC line (HuiPSC). Relative expression values were normalized to GAPDH expression using the ΔCt method. Error bars indicate standard deviation (n = 3 independent passages). **E**: Expression of ectodermal (PAX6, DCX), mesodermal (TBXT) and endodermal (AFP, SOX7) genes was increased in embryoid bodies derived from LEIi010-A and LEIi010-B, compared with undifferentiated iPSC. Relative expression values were normalized to GAPDH expression and fold change in expression calculated using the ΔΔCt method. Error bars indicate standard deviation (n = 3 independent EB cultures). **F**: Heatmap representations of DNA fingerprinting by short tandem repeat (STR) analysis demonstrate both LEIi010-A and LEIi010-B line matched the donor fibroblast line at 16 loci, indicating the iPSC were derived from the patient fibroblasts. **G**: Agarose gel showing mycoplasma PCR screening results for LEIi010-A (A) and LEIi010-B (B). A 259bp mycoplasma specific band was amplified only from the positive control sample (+). The 481bp internal control band was amplified by the negative control sample (-), LEIi010-A (A) and LEIi010-B (B) indicating both lines were negative for mycoplasma. **H**: Agarose gel showing episomal plasmid PCR screening results for LEIi010-A (A) and LEIi010-B (B). Both lines were negative for episomal plasmids, while a 95bp band was amplified in a positive control consisting of genomic DNA from fibroblasts transfected with reprogramming episomes (+). The 95bp episomal plasmid product was not present in a negative control containing no template DNA (-).

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
LEIi010-A	1011ips1	Female	18	Caucasian	*USH2A* c.[949C > A]; [1256G > T]	Usher syndrome type-2
LEIi010-B	1011ips2	Female	18	Caucasian	*USH2A* c.[949C > A]; [1256G > T]	Usher syndrome type-2

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal iPSC morphology	Fig. 1 panel A
Phenotype	Qualitative analysis: Immunocytochemistry	Positive for the pluripotency markers: OCT4, NANOG, SOX2 and SSEA4	Fig. 1 panel C
	Quantitative analysis: RT-qPCR	Positive for the pluripotency markers *OCT4*, *NANOG*, *SOX2*, *KLF4*	Fig. 1 panel D
Genotype	Karyotype (Illumina Beadchip SNP Array with CNV Analysis) and resolution	46XX Resolution: 0.5 Mb	Fig. 1 panel B
Identity	STR analysis	DNA Profiling by STR analysis performed	Results table archived with journal
		Matched at 16 loci	Fig. 1 panel F
Mutation analysis (IF APPLICABLE)	Sequencing	Compound heterozygous for c.949C > T and c.1256G > T in *USH2A*	Fig. 1 panel A
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR assay: Negative	Fig. 1 panel G
Differentiation potential	Embryoid body formation	Upregulation of markers of ectoderm (*PAX6*, *DCX*), mesoderm (TBXT) and endoderm (*AFP, SOX7*)	Fig. 1 panel E
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

Microsatellite analysis

Analysis of microsatellites was performed using the Promega PowerPlex 16HS system (Promega, performed at the Australian Genome Research Facility). Donor fibroblasts and derived iPSC lines were genotyped at the loci D18S51, D21S11, TH01, D3S1358, Penta E, FGA, TPOX, D8S1179, vWA, CSF1PO, D16S539, D7S820, D13S317, D5S818, Penta D and Amelogenin. Results are shown as a heatmap, demonstrating matching genotypes at all loci examined.

Mycoplasma testing

Mycoplasma testing was performed using the Lookout Mycoplasma PCR Detection Kit (Sigma-Aldrich).

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Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Mouse anti-OCT3/4	1:200	Santa Cruz Biotechnology Cat# sc-5279, RRID:AB_628051
	Rabbit anti-SOX2	1:200	Thermo Fisher Scientific Cat# 48-1400, RRID:AB_2533841
	Rabbit anti-NANOG	1:100	Abcam Cat# ab21624, RRID:AB_446437
	Mouse anti-SSEA4	1:100	STEMCELL Technologies, Cat# 60062, RRID:AB_2721031
Secondary antibodies	Alexa Fluor 546 Goat anti-mouse	1:500	Molecular Probes Cat# A-11003, RRID:AB_141370
	Alexa Fluor 488 Goat anti-rabbit	1:500	Molecular Probes Cat# A-11008, RRID:AB_143165
Primers			
	Target	Forward/Reverse primer (5′-3′)	
Episomal Plasmid PCR	Reprogramming plasmids	CTTCAACCATCAGGCTTACTTCTA/ CTGGTGGGTCAGTAACATCATC	
Pluripotency Markers (qPCR)	*SOX2*	GCTACAGCATGATGCAGGACCA/ TCTGCGAGCTGGTCATGGAGTT	
	OCT4	CCTGAAGCAGAAGAGGATCACC/ AAAGCGGCAGATGGTCGTTTGG	
	NANOG	CTCCAACATCCTGAACCTCAGC/ CGTCACACCATTGCTATTCTTCG	
	KLF4	CATCTCAAGGCACACCTGCGAA/ TCGGTCGCATTTTTGGCACTGG	
Ectoderm Markers (qPCR)	*PAX6*	CTGAGGAATCAGAGAAGACAGGC/ ATGGAGCCAGATGTGAAGGAGG	
	DCX	TGCCTCAGGGAGTGCGTTA/ GAACAGACATAGCTTTCCCCTTC	
Endoderm Markers (qPCR)	*SOX7*	TCGACGCCCTGGATCAACT/ CTGGGAGACCGGAACATGC	
	AFP	TGAGCACTGTTGCAGAGGAG/ TTGTTTGACAGAGTGTCTTGTTGA	
Mesoderm Marker (qPCR)	*TBXT*	CCTTCAGCAAAGTCAAGCTCACC/ TGAACTGGGTCTCAGGGAAGCA	
House-Keeping Genes(qPCR)	*GAPDH*	AGAAGGCTGGGGCTCATTTG/ AGGGGCCATCCACAGTCTTC	
Targeted mutation analysis/sequencing	*USH2A* c.949C > A	TGACATTCATTTGTAACGACTCC/ AAGTTTGTGGGCATTTGTTG	
	USH2A c.1256T > C	ATGTTGCTTTTACCACAGGGCT/ AGCCACACTAAAACAGTTCCTTGAA	

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