Stem Cell Research 54 (2021) 102403

Contents lists available at ScienceDirect

Stem Cell Research

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

Lab Resource: Multiple Cell Lines

Generation of two induced pluripotent stem cell lines from a retinitis pigmentosa patient with compound heterozygous mutations in *CRB1*

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A B S T R A C T

Two human iPSC lines were generated from dermal fibroblasts derived from a patient with retinitis pigmentosa caused by *CRB1* mutation using episomal plasmids containing *OCT4*, *SOX2*, *LIN28, KLF4*, *L-MYC* and *mp53DD*. These clonal iPSC lines carry compound heterozygous mutations in *CRB1* (c.2555 T > C and c.3014A > T). Both lines expressed pluripotency markers, displayed a normal karyotype and demonstrated the ability to differentiate into the three primary germ layers, as well as retinal organoids.

Resource Table:

Unique stem cell lines identifier	LEIi016-A LEIi016-B
Alternative name(s) of stem cell lines	1418ips3 (LEIi016-A) 1418ips6 (LEIi016-B)
Institution	The Lions Eye Institute
Contact information of distributor	Dr Samuel McLenachan smclenachan@lei.org.au Dr Fred K Chen fredchen@lei.org.au
Type of cell lines	iPSC
Origin	Human
Additional origin info required	*Age:* 11 *Sex:* Male *Ethnicity if known:* Caucasian
Cell Source	Dermal fibroblasts
Clonality	Clonal
Associated disease	Retinitis Pigmentosa 12
Gene/locus	CRB1/1q31.3
Date archived/stock date	1418ips3 (LEIi016-A) 06/08/2020 1418ips6 (LEIi016-B) 06/08/2020
Cell line repository/bank	https://hpscreg.eu/cell-line/LEIi016-A https://hpscreg.eu/cell-line/LEIi016-B
Ethical approval	University of Western Australia Human Research Ethics Committee RA/4/1/7916

1. Resource utility

Mutations in the *crumbs homolog 1* (*CRB1*) gene are responsible for retinal dystrophies that exhibit a range of disease severities and phenotypes. The reported iPSC lines were generated from a patient with childhood-onset retinitis pigmentosa caused by *CRB1* mutations, providing a valuable resource for *in vitro* modelling of this disease.

2. Resource details

Over 350 pathogenic mutations have been identified in the *CRB1* gene and associated with various retinopathies including maculopathy, retinitis pigmentosa (RP) and Leber congenital amaurosis (Boon et al. 2020). CRB1-related retinopathies are characterised by maculopathy, loss of retinal lamination, increased retinal thickness and preservation of the *para*-arteriolar retinal pigment epithelium. Here, we report the generation of two iPSC lines from a patient with early-onset *CRB1*-associated RP. The male patient presented at age 11 with a history of poor night vision and hyperopic refractive correction from the age 5 years. Optical coherence tomography demonstrated thickening and disorganisation of retinal layers. Multimodal imaging data was reported previously (Roshandel et al. 2021, pedigree 2118). Two substitution mutations in the *CRB1* gene (NM_201253.3) were identified in the patient's DNA by targeted Sanger sequencing: c.2555 T > C (Hasan et al.

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https://doi.org/10.1016/j.scr.2021.102403
Received 3 May 2021; Accepted 15 May 2021
Available online 20 May 2021

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	Normal	Fig. 1, panels A-B
Phenotype	Qualitative analysis: Immunocytochemistry	Positive for the pluripotency markers: OCT4, NANOG, SOX2	Fig. 1, panels A-B
	Quantitative analysis: qPCR	Positive for the pluripotency markers OCT4, NANOG, SOX2, KLF4	Fig. 1, panel E
Genotype	Karyotype (HumanCoreExome-24) and resolution	46,XY 500 kb resolution	Fig. 1, panels C-D
Identity		STR analysis performed	
	STR analysis	Matched at 16 loci	Archived with journal
Mutation analysis (IF APPLICABLE)	Sequencing	*CRB1* c.[2555A > T];[3014 T > C]	Fig. 1, panels A-B
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR: Negative	Fig. 1, panel G
Differentiation potential	Embryoid body formation	Upregulation of ectoderm (*PAX6*, *DCX*), mesoderm (*DCN, IGF2, TBXT, NKX2.5*) and endoderm (*SOX17*, *FOXA2*) markers	Fig. 1, panel F
	Directed differentiation (retinal organoids)	Upregulation of retinal markers (*PAX6*, *CRX*, *RCVRN* and *RHO*)	Fig. 1, panel H
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

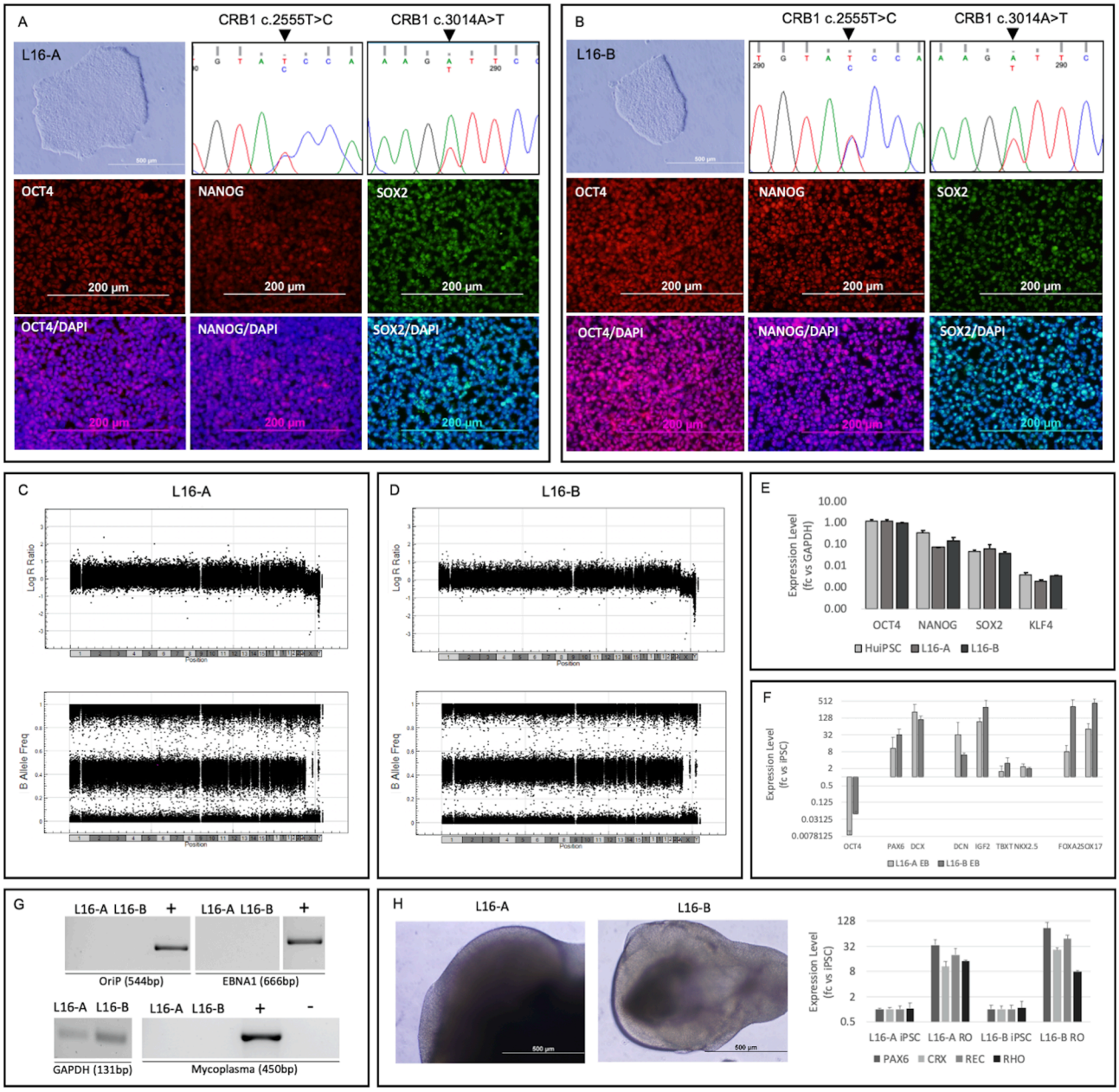

Fig. 1. STR analysis.

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry				
	Antibody	Dilution	Company Cat #	RRID
Pluripotency Markers	Mouse anti-OCT4	1:200	Santa Cruz Biotechnology, sc-5279	AB_628051
	Mouse anti-NANOG	1:1000	Abcam ab21624	AB_446437
	Rabbit anti-SOX2	1:1000	Sino Biological 101284-T42	AB_2810307
Secondary antibodies	Goat anti-Mouse AlexaFluor 546	1:500	Invitrogen A11003	AB_141370
	Goat anti-Rabbit AlexaFluor 488	1:500	Invitrogen A11008	AB_143165
Primers				
	Target	Size of band	Forward/Reverse primer (5′-3′)	
Episomal Plasmids (PCR)	Episomal vectors (*OriP*)	544 bp	TTCCACGAGGGTAGTGAACC/ TCGGGGGTGTTAGAGACAAC	
	Episomal vectors (*EBNA1*)	666 bp	ATCGTCAAAGCTGCACACAG/ CCCAGGAGTCCCAGTAGTCA	
Mycoplasma Primers (PCR)	Mycoplasma DNA	450 bp	YGCCTGVGTAGTAYRYWCGC/ GCGGTGTGTACAARMCCCGA	
Pluripotency Markers (qPCR)	*OCT4*	105 bp	CCTGAAGCAGAAGAGGATCACC/ AAAGCGGCAGATGGTCGTTTGG	
	NANOG	114 bp	CTCCAACATCCTGAACCTCAGC/ CGTCACACCATTGCTATTCTTCG	
	SOX2	134 bp	GCTACAGCATGATGCAGGACCA/ TCTGCGAGCTGGTCATGGAGTT	
	KLF4	110 bp	CATCTCAAGGCACACCTGCGAA/ TCGGTCGCATTTTTGGCACTGG	
Trilineage Markers (qPCR)	*PAX6*	130 bp	CTGAGGAATCAGAGAAGACAGGC/ ATGGAGCCAGATGTGAAGGAGG	
	DCX	94 bp	TGCCTCAGGGAGTGCGTTA/ GAACAGACATAGCTTTCCCCTTC	
	TBXT	152 bp	CCTTCAGCAAAGTCAAGCTCACC/ TGAACTGGGTCTCAGGGAAGCA	
	NKX2.5	146 bp	AAGTGTGCGTCTGCCTTTCCCG/ TTGTCCGCCTCTGTCTTCTCCA	
	FOXA2	133 bp	GGAACACCACTACGCCTTCAAC/ AGTGCATCACCTGTTCGTAGGC	
	SOX17	111 bp	ACGCTTTCATGGTGTGGGCTAAG/ GTCAGCGCCTTCCACGACTTG	
Retinal Markers	*PAX6*	130 bp	CTGAGGAATCAGAGAAGACAGGC/ ATGGAGCCAGATGTGAAGGAGG	
	CRX	128 bp	CCAGTGTGGATCTGATGCACCA/ GGTACTGGGTCTTGGCAAACAG	
	RCVRN	90 bp	CCAGAGCATCTACGCCAAGTT/ CCGTCGAGGTTGGAATCGAAG	
	RHO	122 bp	AGCTCGTCTTCACCGTCAAGGA/ CCAGCAGATCAGGAAAGCGATG	
House-Keeping Genes (qPCR)	*GAPDH*	131 bp	GTCTCCTCTGACTTCAACAGCG/ ACCACCCTGTTGCTGTAGCCAA	
Targeted mutation sequencing	*CRB1* c.2555A > T	563 bp	AACGCTTCAACCATCAGGCT/ TACTGGTGGGTCAGTAACATCAT	
	CRB1 c.3014 T > C	440 bp	AAAAGCAACTAGCACAGTATGTAAC/ GGACAGTGGGTCTGTCATGG	

2016) and c.3014A > T (Corton et al. 2013). Analysis of parental DNA confirmed independent segregation of the two variants.

Dermal fibroblasts derived from the patient were transfected with iPSC reprogramming episomes. After 25 days, two clonal iPSC lines (LEIi016-A and -B) were selected for expansion and characterization (Table 1). Both lines displayed typical iPSC colony morphology (Fig. 1A-B, brightfield micrographs). The presence of the c.2555 T > C and c.3014A > T variants was confirmed in LEIi016-A and -B by PCR and Sanger sequencing (Fig. 1A-B, chromatograms). Immunostaining demonstrated the expression of the pluripotency markers OCT4, NANOG and SOX2 in LEIi016-A (passage 26) and -B (passage 27) iPSC (Fig. 1A-B, fluorescence micrographs). Digital karyotyping of LEIi016-A and -B was performed at passage 11 using an Illumina Beadchip assay, followed by copy number variation analysis. LogR and B allele frequencies were plotted against genomic location (Fig. 1C-D), demonstrating a 46,XY karyotype. Quantitative polymerase chain reaction (qPCR) analyses showed similar expression of *OCT4*, *NANOG*, *SOX2* and *KLF4* in the LEIi016-A (passage 15–17) and -B (passage 14–16) iPSC lines and a commercial human iPSC line (Fig. 1E). The trilineage differentiation potential of the LEIi016-A and -B iPSC lines was demonstrated by upregulation of markers for ectoderm (*PAX6*, *DCX*), mesoderm (*DCN*, *IGF2, TBXT and NKX2.5*) and endoderm (*FOXA2, SOX17*) and down-regulation of the pluripotency marker *OCT4* after two weeks of differentiation as embryoid bodies (EBs, Fig. 1F). PCR analysis using two sets of episome specific primers (targeting *OriP* or *EBNA1*) demonstrated both iPSC lines were negative for reprogramming episomes after passage 25. Episome specific bands (544 bp and 666 bp) were detected in a positive control consisting of passage 1 iPSCs (Fig. 1G). PCR analysis demonstrated that LEIi016-A (passage 27) and -B (passage 26) were negative for mycoplasma DNA, whereas a 450 bp band was detected in the positive control sample (Fig. 1G). A 131 bp GAPDH band was detected in gDNA samples from both lines. No bands were amplified in negative controls reactions without template DNA (Fig. 1G). After two months of directed retinal differentiation, both the LEIi016-A and -B iPSC lines produced retinal organoids. Expression of retinal marker genes was measured by qPCR, demonstrating upregulation of *PAX6*, *CRX*, *RCVRN* and *RHO* in retinal organoid cultures, compared with undifferentiated iPSC (Fig. 1H).

3. Materials and methods

Ethics: This work was approved by the University of Western

Australia Human Research Ethics Committee (RA/4/1/7916) with written consent from the patient, and carried out in accordance with the National Health & Medical Research Council of Australia National Statement on Ethical Conduct in Human Research (2007, updated 2018) and the Declaration of Helsinki.

Cell culture: Fibroblasts were cultured in DMEM medium with 10% fetal calf serum (26140079, ThermoFisher) and antibiotic–antimycotic (15240096, ThermoFisher). Fibroblasts were reprogrammed using the Epi5 Episomal iPSC Reprogramming System (A15960, ThermoFisher). 100,000 cells were electroporated using the NEON Transfection System (ThermoFisher, 3x10ms 1650 V pulses, 10 µl tip). Colonies were picked on day 25. Colonies were dissociated into cell clusters with TrypLE (12604021, ThermoFisher) every 4–5 days and passaged at a ratio of 1:3–1:6 onto geltrex (A1413202, ThermoFisher) coated plates in mTeSR Plus medium (100–0276, StemCell Technologies). EBs were differentiated by seeding iPSC clusters into suspension culture plates in mTeSR Plus media with 10 µM Y27632 (ab120129, Abcam). Medium was changed after 48 h to DMEM/F12 (11320033, ThermoFisher) with 20% knockout serum replacement (KSR, A3181502, ThermoFisher), non-essential amino acids (11140050, ThermoFisher) and antibiotic-antimycotics. EB RNA was harvested on day 14. Retinal organoid differentiation was performed as previously described (Zhang et al. 2020). Cells were cultured at 37 °C with 5%CO_2.

RNA analysis: RNA was isolated using TRIZOL and cDNA synthesised using the RT^{2}First Strand Kit (330404, Qiagen). qPCR was performed with RT^{2}SYBR Green qPCR Mastermix (330503, Qiagen) on the CFX Connect Real-Time System (BioRad, 45 cycles, 95 °C for 20 s, 60 °C for 60 s). Expression values were normalized against *GAPDH* expression (ΔΔCT method). Primers are listed in Table 2.

Immunostaining analysis: iPSCs were fixed with 4% paraformaldehyde at 37 °C for 10 min, washed with phosphate buffered saline (PBS, 10010023, ThermoFisher) then incubated for 10 min in chilled methanol. The cells were incubated in blocking buffer (BB, 0.3% Triton X-100 and 5% normal goal serum in PBS) for 1 h at room temperature. Primary antibodies were diluted in BB and applied at 4 °C overnight. Secondary antibodies were diluted in BB and applied for 2 h at room temperature. Nuclei were stained with DAPI. Antibodies are listed in Table 2.

DNA analyses: DNA was extracted using the FlexiGene DNA Kit (51206, QIAGEN). Digital karyotyping was performed by the Australian Genome Research Facility, (AGRF, Perth, Western Australia), using the Infinium HumanCoreExome-24 Beadchip SNP array (Illumina, San Diego, California, United States), followed by copy number variation analysis using GenomeStudio 2.0 software with the CNVpartition 2.0 plugin (Illumina). Microsatellite analysis was performed by AGRF using the Powerplex16H system (Promega, Madison, Wisconsin, United States). PCR for Sanger sequencing was performed on a BioRad T100 Thermal Cycler (35 cycles, 98 °C for 10 s, 60 °C for 30 s, 72 °C for 45 s). PCR screening for episomes was performed using two primer sets according to the Epi5 Episomal iPSC Reprogramming System protocol. Mycoplasma testing was conducted using the HotStarTaq Master Mix Kit (203443, Qiagen) using redundant primer sequences targeting 12 mycoplasma strains (Table 2) and the pIDTSMART-AMP:MPCon (Ref ID: 96692130) plasmid a positive control.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work was funded by the National Health and Medical Research Council of Australia (GNT1188694, GNT1054712, MRF1142962), the Ophthalmic Research Institute Australia, the McCusker Charitable Foundation and generous donations from the Constantine and Saleeba families.

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