Stem Cell Research 54 (2021) 102448

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 patient with Stargardt disease caused by compound heterozygous mutations in the ABCA4 gene

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A B S T R A C T

Stargardt disease (STGD1) is the most common inherited retinal dystrophy and *ABCA4* c.546−10 T>C is the most commonly reported splice mutation. Here, we generated and characterized two induced pluripotent stem cell (iPSC) lines from a STGD1 patient with compound heterozygous mutations in *ABCA4* (c.[5461-10 T > C;5603A > T];[4163 T > C;455G > A]). Episomal vectors containing *OCT4, SOX2, KLF4, L-MYC, LIN28* and *mp53DD* were employed to conduct the reprogramming of patient-derived fibroblasts. Both lines had a normal karyotype, displayed iPSC morphology, expressed pluripotency markers and showed trilineage differentiation potential. These lines can provide a powerful platform for further investigating the pathophysiological consequences of mutations in *ABCA4*.

1. Resource Table:

Unique stem cell lines identifier	LEIi017-A LEIi017-B
Alternative name(s) of stem cell lines	1629ips9 (LEIi017-A) 1629ips11 (LEIi017-B)
Institution	Lions Eye Institute
Contact information of distributor	Samuel McLenachan: smclenachan@lei.org.au Fred K. Chen: fredchen@lei.org.au
Type of cell lines	iPSC
Origin	Human
Additional origin info required for human ESC or iPSC	Age: 16 Sex: Female Ethnicity if known: Caucasian
Cell Source	Dermal fibroblasts
Clonality	Clonal
Associated disease	Stargardt disease
Gene/locus	*ABCA4*/1p22.1
Date archived/stock date	Both lines 24/06/2020
Cell line repository/bank	https://hpscreg.eu/cell-line/LEIi017-A https://hpscreg.eu/cell-line/LEIi017-B
Ethical approval	University of Western Australia Human Research Ethics Committee RA/4/1/7916

2. Resource utility

Autosomal recessive Stargardt disease is the most common form of juvenile-onset macular degeneration and is caused by mutations in the *ABCA4* gene (Allikmets et al., 1997). The LEIi017-A and LEIi017-B lines generated from a patient carrying compound heterozygous *ABCA4* mutations will provide a powerful resource for investigating the effects of *ABCA4* mutations.

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https://doi.org/10.1016/j.scr.2021.102448
Received 9 June 2021; Accepted 21 June 2021
Available online 24 June 2021

3. Resource details

Stargardt disease (STGD1, OMIM:248200) is the most common retinal dystrophy featured by progressive central vision loss (Huang et al., 2020). Mutations in the *ATP-binding cassette transporter* gene (*ABCA4*) have been associated with heterogeneous retinal phenotypes ranging in severity from early-onset cone-rod dystrophy to late-onset macular dystrophy (Allikmets et al., 1997; Maugeri et al., 2000; Cremers et al., 1998). Here, we report the generation and characterization of two iPSC lines from a patient diagnosed with STGD1 caused by compound heterozygous mutations in *ABCA4* (c.[5461-10 T > C;5603A > T]; [4163 T > C;455G > A]).

The female patient presented with reduced vision at the age of 6 years without retinal lesions. Electroretinography at age 9 demonstrated an electronegative waveform. Stargardt disease was suspected at age 15 when flecks were first noted. By age 16, her visual acuity had dropped to 6/120 in both eyes. Genetic analysis using the Stargardt/Macular dystrophy panel (version 3; 10 genes) identified four mutations in *ABCA4* (NM_000350.2). Phase analysis was performed by targeted Sanger sequencing of parental DNA, demonstrating the maternal complex allele carries c.[5461–10 T > C;5603A > T] and the paternal complex allele carries c.[4163 T > C;455G > A]. Dermal fibroblasts were sourced and cultured from the patient (Huang et al., 2020). Reprogramming of fibroblasts into iPSC was conducted using episomal plasmids encoding *OCT4*; *SOX2*, *KLF4*, *L-MYC*, *LIN28* and *mp53DD*. Two clonal iPSC lines (LEIi017-A and LEIi017-B) were established for further characterization (Table 1). Both LEIi017-A and LEIi017-B displayed morphology typical of pluripotent stem cell colonies (Fig. 1A). The expression of pluripotency markers (OCT4, NANOG, SOX2 and KLF4) was demonstrated by immunocytochemistry at passage 33, and the cell nuclei were stained with DAPI (Fig. 1A). All *ABCA4* mutations detected in the patient were confirmed in both LEIi017 iPSC lines by Sanger sequencing (Fig. 1B). Quantitative reverse transcription polymerase chain reaction (RT-qPCR) demonstrated *OCT4*, *NANOG*, *SOX2*, *KLF4* and *MYC* mRNA was expressed comparably in LEIi017-A and LEIi017-B (passage 11), and in a control human iPSC line (HuiPSC, Fig. 1C). The capacity of LEIi017-A and LEIi017-B to differentiate into three germ layers was confirmed by the expression of markers of ectoderm (*PAX6*, *OTX1*), mesoderm (*TBXT*, *NKX2.5*) and endoderm (*AFP*, *SOX17*) lineages in differentiating embryoid body (EB) cultures by RT-qPCR. After 14-day spontaneous differentiation, EBs derived from LEIi017-A (passage 35) and LEIi017-B (passage 34) increased expression of ectoderm, mesoderm and endoderm markers, compared with undifferentiated iPSC (Fig. 1D). Digital karyotyping using the Infinium Human CoreExome-24 Beadchip SNP array (Illumina, San Diego, California, United States) 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 and LogR ratios of ≈500,000 single nucleotide polymorphisms located across the genome demonstrated an absence of reprogramming-induced chromosomal rearrangements in LEIi017-A and LEIi017-B (passage 12 for both, Fig. 1E). Genotyping of 16 microsatellite markers confirmed both iPSC lines were derived from the patient's fibroblasts (data not shown). LEIi017-A (passage 12) and LEIi017-B (passage 12) were negative for reprogramming episomes and mycoplasma (Fig. 1F). The 130 bp *GAPDH* internal control product was amplified from LEIi017-A and LEIi017-B (Fig. 1F).

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	Normal	Fig. 1, panels A
Phenotype	Qualitative analysis: Immunocytochemistry	Positive for the pluripotency markers: OCT4, NANOG, SOX2, KLF4	Fig. 1, panels A
	Quantitative analysis: RT-qPCR)	Positive for the pluripotency markers *OCT4, NANOG, SOX2, KLF4, C-MYC*	Fig. 1, panel C
Genotype	Karyotype (the Infinium HumanCytoSNP-12 Beadchip SNP array) and resolution	46,XX Resolution 500 kb	Fig. 1, panels E
Identity		STR analysis performed	
	STR analysis	Matched at 16 loci	Archived with journal
Mutation analysis (IF APPLICABLE)	Sequencing	*ABCA4* c.[5461–10 T > C;5603A > T]; [4163 T > C;455G > A]	Fig. 1, panels B
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR: Negative	Fig. 1, panel F
Differentiation potential	Embryoid body formation	Upregulation of ectoderm (*PAX6, OTX1*), mesoderm (*TBXT, NKX2.5*) and endoderm (*AFP, SOX17*) markers	Fig. 1, panel D
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping HLA tissue typing	N/A N/A	N/A N/A

4. Materials and methods

Ethics: This work was approved by the University of Western Australia Human Research Ethics Committee (RA/4/1/7916). Written consent was obtained from the patient and all procedures complied with requirements of 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 (11995065, Gibco) supplemented with 10% fetal bovine serum (FBS, 26140079, Gibco) and 1 × Antibiotic-Antimycotic (15240062, Gibco). Human iPSC lines were cultured on Geltrex-coated (A1413202, Gibco) plates in TeSR-E8 medium (05990, StemCell Technologies) and passaged using TrypLE Express Enzyme (12604021, Gibco) every 4–5 days using a split ratio of 1:3–1:6. Both fibroblasts and iPSC cultures were maintained at 37 °C with 5% CO_2.

Reprogramming: Fibroblasts were reprogrammed using the Epi5™ Episomal iPSC Reprogramming Kit (A15960, Invitrogen). Fibroblast cells (1×10^5) were electroporated using the Invitrogen NEON electroporation system (three 10 ms pulses at 1650 V in a 10 μl tip). iPSC colonies were picked for clonal expansion on day 28.

Trilineage differentiation: EB differentiation was performed by seeding iPSC clusters into 6-well suspension plates containing mTesR1 medium (85850, StemCell Technologies) and 10 μM Y27632 (ab120129, Abcam). On day 3, medium was changed to DMEM/F12 (11320033, Gibco) supplemented with 20% knockout serum replacement (KSR, 10828–028, Gibco), Antibiotic–Antimycotic and MEM-non essential amino acids (MEM-NEAA, 11140050,Gibco). Half medium changes were performed daily and EB RNA was harvested at day 14.

RNA analysis: RNA extraction was performed using MagMAX TM-96 Total RNA Isolation kit (AM1830, Invitrogen). RT-qPCR was performed with RT^2 SYBR Green qPCR Mastermix (330503, Qiagen) on the BioRad CFX Connect Real-Time System (45 cycles, 95 °C for 30 s, 60 °C for 60 s). Gene expression was quantified using the ΔCT method, normalized to *GAPDH* expression. Primers used are listed in Table 2.

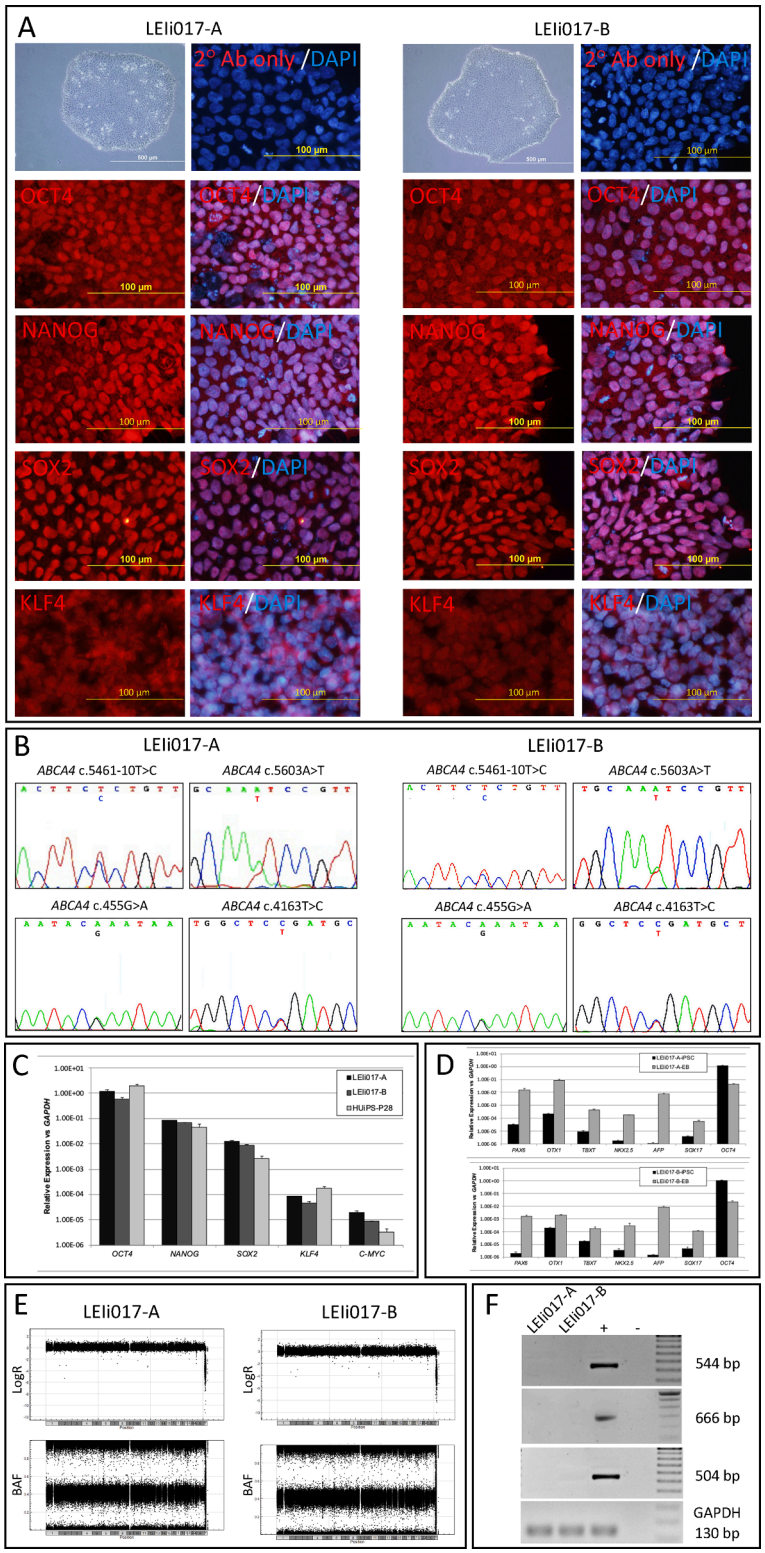

Fig. 1. Characterization of LEIi017-A and LEIi017-B iPSC. (**A**) LEIi017-A and LEIi017-B iPSC colonies showed typical pluripotent stem cell morphology (brightfield images) and were positive for OCT4, NANOG, SOX2 and KLF4 immunostaining (red signal, immunofluorescence images). Counterstaining with DAPI (blue signal) demonstrated nuclear localization of each marker (merged fluorescence images). Negative controls with primary antibodies omitted (2° Ab only) showed no background staining. (**B**) Sanger sequencing chromatograms confirmed the presence of the c.[5461-10T>C;5603A>T] and c.[4163T>C;455G>A] complex alleles in LEIi017-A and LEIi017-B. (**C**) Quantitative PCR analysis demonstrated similar levels of *OCT4*, *NANOG*, *SOX2*, *KLF4* and *C-MYC* mRNA expression in LEIi017-A, LEIi017-B and a commercial control iPSC line (HUiPS P28). (**D**) Quantitative PCR analysis demonstrated upregulation of ectoderm (*PAX6*, *OTX1*), mesoderm (*TBXT*, *NKX2.5*) and endoderm (*AFP*, *SOX17*) markers and downregulation of *OCT4* in embryoid bodies (EB) derived from LEIi017-A (upper panel) and LEIi017-B (lower panel), compared with undifferentiated iPSC. (**E**) LogR and B allele frequency (BAF) scatter plots of » 500,000 human genomic SNPs demonstrated a normal 46,XX karyotype in both LEIi017-A and LEIi017-B. (**F**) PCR analysis demonstrated LEIi017-A and LEIi017-B were negative for reprogramming episomes (544bp and 666bp) and mycoplasma DNA (504bp) and positive for *GAPDH* (130bp) at passage 12. Positive controls (+) showed amplification of the expected bands. No bands were amplified in negative controls without template DNA (-).

Immunocytochemistry analysis: Patient iPSCs were fixed with 4% paraformaldehyde for 15 min at 37°C, washed with PBS and incubated with blocking buffer (5% normal goat serum in PBS containing 0.3% Triton X100) for 1 h at room temperature, followed by incubation with primary antibodies at 4°C overnight. Negative controls omitting primary antibodies were included. Secondary antibodies and DAPI (1 µg/mL) were applied for 1 h at room temperature. Antibodies are listed in Table 2.

Digital karyotyping and microsatellite analysis: Karyotyping was performed on LEIi017-A and LEIi017-B by the Australian Genome Research Facility (AGRF, Perth, Western Australia), using the Infinium Human CoreExome-24 Beadchip SNP array (Illumina, United States). CNV analysis was performed using GenomeStudio 2.0 software with the cnvPartition 2.0 plugin (Illumina). Microsatellite analysis was performed by AGRF using the Promega Powerplex 16H system (Promega, United States).

Table 2
Reagents details.

	Antibodies used for immunocytochemistry			
	Antibody	Dilution	Company Cat #	RRID
Pluripotency Markers	Rabbit anti-OCT4	1:1000	Sino biological 101282-T02	AB_2810309
	Rabbit anti-NANOG	1:1000	Sino biological 101286-T34	AB_2810308
	Rabbit anti-SOX2	1:1000	Sino Biological 101284-T42	AB_2810307
	Rabbit anti-KLF4	1:100	Invitrogen 710,659	AB_2532749
Secondary antibodies	Goat anti-Rabbit AlexaFluor 546	1:250	Invitrogen A11035	AB_2534093
	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	504 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	
	MYC	128 bp	CCTGGTGCTCCATGAGGAGAC/ CAGACTCTGACCTTTTGCCAGG	
Trilineage Markers (qPCR)	*PAX6*	130 bp	CTGAGGAATCAGAGAAGACAGGC/ ATGGAGCCAGATGTGAAGGAGG	
	OTX1	159 bp	CTACCCTGACATCTTCATGCGG/ GGAGAGGACTTCTTCTTGGCTG	
	TBXT	152 bp	CCTTCAGCAAAGTCAAGCTCACC/ TGAACTGGGTCTCAGGGAAGCA	
	NKX2.5	146 bp	AAGTGTGCGTCTGCCTTTCCCG/ TTGTCCGCCTCTGTCTTCTCCA	
	AFP	96 bp	TGAGCACTGTTGCAGAGGAG/ TTGTTTGACAGAGTGTCTTGTTGA	
	SOX17	111 bp	ACGCTTTCATGGTGTGGGCTAAG / GTCAGCGCCTTCCACGACTTG	
House-Keeping Genes (qPCR)	*GAPDH*	130 bp	GTCTCCTCTGACTTCAACAGCG/ ACCACCCTGTTGCTGTAGCCAA	
Targeted mutation sequencing	*ABCA4*	765 bp	CCTTGAGGCACTGCTTGTAAG/ GGTCAGGAGGAAGTACACCAC	
	ABCA4	585 bp	CCATCCAGCCCTGGCTAACT/ GGTCACCAGGATGTGTACAGC	
	ABCA4	442 bp	TGAAGTCTGGCTGAACACTG/ CTTCTAAGCAGCATGTGACC	

DNA Analysis: Genomic DNA was extracted using FlexiGene DNA kit (51206, Qiagen). PCR for Sanger sequencing was performed on a GeneTouch Thermal Cycler (35 cycles, 94 °C for 30 s, 60 °C for 60 s, 72 °C for 2mins). Mycoplasma testing by PCR was performed using the Mycoplasma Testing Kit (#091, Media and Monoclonal Laboratories, Harry Perkins Institute of Medical Research, Fig. 1F). PCR screening for reprogramming episomes was performed using primers listed in Table 2.

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.

Acknowledgements

This work was funded by the National Health and Medical Research Council of Australia (GNT1188694, MRF1142962 and GNT1116360), the Telethon Perth Children's Hospital Research Fund 2016 (Round 5), the McCusker Charitable Foundation and the Macular Disease Foundation Australia Research Grant 2018, as well as generous donations from the Saleeba, Constantine and Miocevich families. PhD Scholarship of the first author is awarded by the Perron Institute and Murdoch University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2021.102448.

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