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Lab Resource: Multiple Cell Lines

Generation of two induced pluripotent stem cell lines from a patient with Stargardt Macular Dystrophy caused by the c.768G > T and c.6079C > T mutations in *ABCA4*

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A B S T R A C T

Autosomal recessive Stargardt disease is the most common cause of inherited retinal disease. In this report, we describe the generation and characterization of two human induced pluripotent stem cell (iPSC) lines from a patient with compound heterozygous mutations in the *ABCA4* gene (c.[768G > T];[6079C > T]). Patient dermal fibroblasts were reprogrammed using episomal plasmids encoding *OCT4*, *SOX2*, *KLF4*, *L-MYC*, *LIN28*, mir302/367 microRNA and shRNA for *P53*. The clonal iPSC lines LEIi012-A and LEIi012-B were established. Both lines had a normal karyotype, displayed iPSC morphology, expressed pluripotency genes at similar levels to control iPSC and displayed trilineage differentiation potential during embryoid body differentiation.

1. Resource table

Unique stem cell lines identifier	LEIi012-A LEIi012-B
Alternative names of stem cell lines	1357iPS5 1357iPS11
Institution	The University of Western Australia
Contact information of distributor	Dr Samuel McLenachan: smclenachan@lei.org.au Dr. Fred Chen: fredchen@lei.org.au
Type of cell lines	iPSC
Origin	Human
Cell Source	Dermal fibroblasts 31 Male Caucasian
Clonality	Clonal
Method of reprogramming	Episomal vectors
Multiline rationale	Isogenic clones
Gene modification	Yes
Type of modification	Hereditary
Associated disease	Stargardt Macular Dystrophy
Gene/locus	*ABCA4*/1p22.1
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	20/03/2017
Cell line repository/bank	https://hpscreg.eu/cell-line/LEIi012-A https://hpscreg.eu/cell-line/LEIi012-B
Ethical approval	Human Research Ethics Office, University of Western Australia (RA/4/1/7916)

2. Resource utility

Autosomal recessive Stargardt disease (STGD1) is a form of juvenile macular degeneration caused by mutations in the *ABCA4* gene. The LEIi012-A and LEIi012-B iPSC lines were generated from a patient harbouring compound heterozygous *ABCA4* mutations, providing a valuable resource for investigating the molecular mechanisms of STGD1.

3. Resource details

Autosomal recessive Stargardt disease (STGD1; OMIM#248200) is the most common inherited retinal disease with a prevalence of 1 in 8000–10,000 (Tanna et al., 2017). STGD1 patients experience a progressive loss of their central visual field caused by mutations in the *ABCA4* gene (Tanna et al., 2017). Here, we have generated two iPSC

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Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
LEIi012-A	012-A	Male	31	Caucasian	c.768G > T c.6079C > T	Stargardt Macular Dystrophy
LEIi012-B	012-B	Male	31	Caucasian	c.768G > T c.6079C > T	Stargardt Macular Dystrophy

lines from a 31-year old male patient with STGD1. The patient presented with retinal flecks and visual acuity of 6/12 R and 6/9 L at age 16. Genetic screening of patient DNA using the ABCR Microarray version 11 (Asper Ophthalmics, Estonia) revealed two pathogenic variants in *ABCA4* (NM_000350.-2), including the non-canonical splice site (NCSS) mutation c.768G > T (Sangermano et al., 2018) and the c.6079C > T missense mutation (Garces et al., 2018). Targeted Sanger sequencing was performed to demonstrate independent segregation of each variant in family members. NCSS mutations alter pre-mRNA splicing of *ABCA4* and account for 13.6% (Fadaie et al., 2019) of the > 900 pathogenic *ABCA4* variants (Tanna et al., 2017). The c.6079C > T mutation causes an L2027F amino acid change within the second ATP-binding domain of the ABCA4 protein, which reduces ATP's binding affinity for ABCA4 protein (Garces et al., 2018).

Patient-derived dermal fibroblasts were obtained from a skin biopsy and cultured in the laboratory. Fibroblasts were reprogrammed using episomal plasmids expressing *OCT4*, *SOX2*, *KLF4*, *L-MYC*, *LIN28*, the miR302/367 cluster and shRNA for *P53*. The clonal iPSC lines LEIi012-A and LEIi012-B were established (Table 1) and characterised to confirm pluripotency (Table 2). Both lines displayed typical iPSC colony morphology (Fig. 1A, B). PCR and Sanger sequencing confirmed the c.768G > T and c.6079C > T mutations were present in a heterozygous state in both lines (Fig. 1A, B). Microsatellite marker analysis confirmed LEIi012-A and LEIi012-B were derived from the patient's dermal fibroblasts (data not shown). Copy number variation (CNV) profiling demonstrated that both LEIi012-A and LEIi012-B had normal diploid karyotypes and were male (46, XY). Analysis of the B allele frequencies and LogR ratios of 300,000 single nucleotide polymorphisms located across the genome demonstrated the absence of chromosomal alterations (Fig. 1C, D). Protein expression of the pluripotency markers OCT4, NANOG and SOX2 was demonstrated in LEIi012-A and LEIi012-B by immunostaining (Fig. 1E, F). Pluripotency markers in both patient lines showed similar mRNA expression levels as a commercially available control human iPSC line (HuiPSC) when measured using quantitative real time polymerase chain reaction (qPCR) (Fig. 1G). Both LEIi012-A and LEIi012-B demonstrated trilineage differentiation potential, with upregulation of ectoderm (*PAX6, OTX1*), mesoderm (*GATA2*, *IGF2*) and endoderm (*AFP*) lineage markers and downregulation of *OCT4* in iPSC-derived embryoid bodies (Fig. 1H). LEIi012-A and LEIi012-B tested negative for mycoplasma (Supplementary Fig. S1A). The 481 bp internal control amplicon was observed in both clonal patient lines and the negative control sample, while the mycoplasma-specific 260 bp band was amplified in the positive control. Reprogramming episomes were not detected in LEIi012-A and LEIi012-B after passage 15, but were detected in the positive control 1020iPS after passage 3 (Supplementary Fig. S1B).

4. Materials and methods

4.1. Ethics

Patient DNA was obtained and analyzed by the Australian Inherited Retinal Disease Registry and DNA Bank, as approved by the Sir Charles Gairdner Hospital Human Research Ethics Committee (2001–053). The collection of patient samples and subsequent iPSC generation 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 were carried out in accordance with the requirements of the National Health & Medical Research Council of Australia and the Declaration of Helsinki.

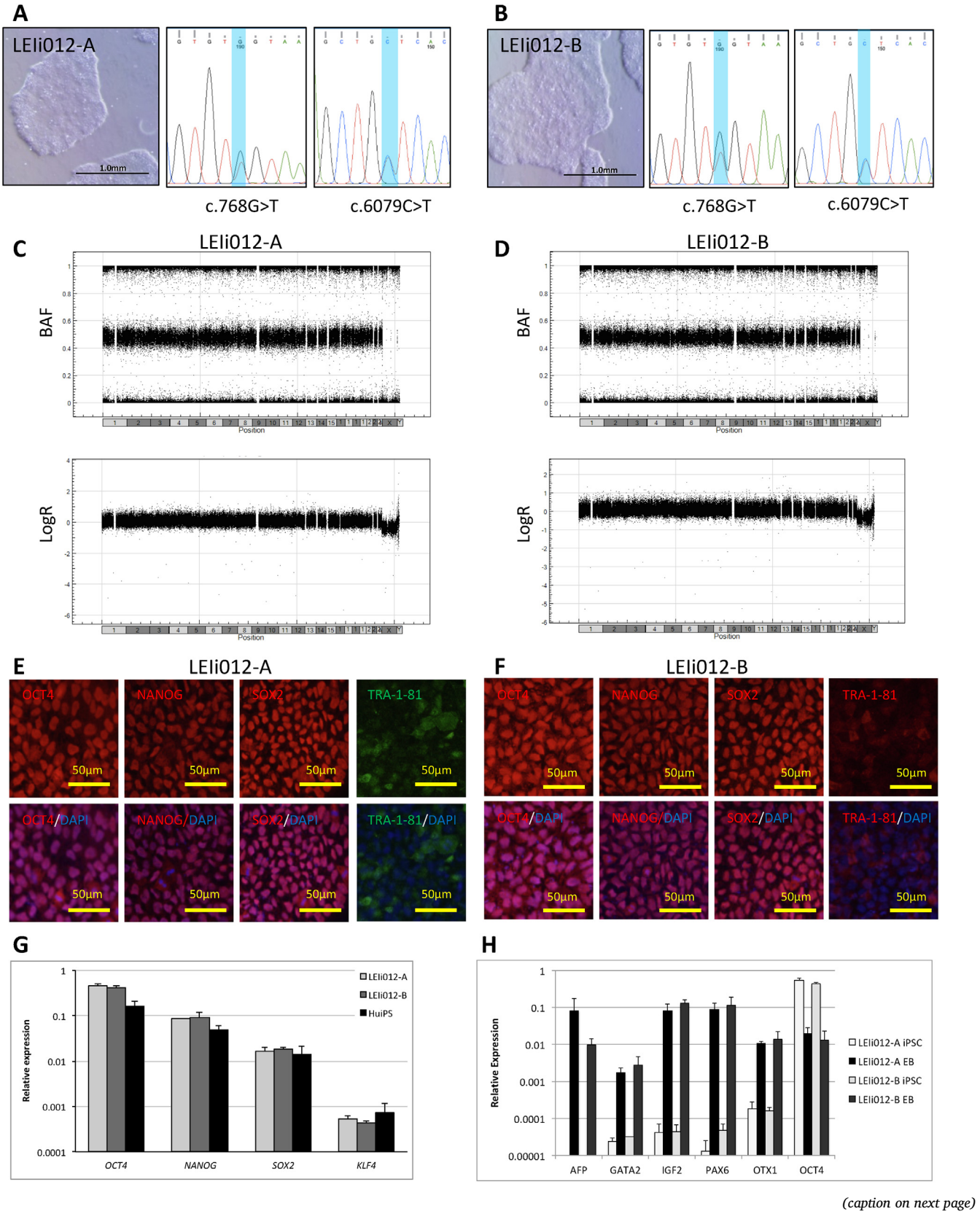
4.2. Cell culture

Patient fibroblasts were cultured in DMEM medium supplemented with 10% fetal calf serum (ThermoFisher). Fibroblasts were passaged once per week using a split ratio of 1:5. Upon reaching 80–90% confluence, media was removed and fibroblast monolayers were rinsed in PBS then incubated with TrypLE (ThermoFisher) for 5 min at 37 °C. An equal volume of culture media was then added to the suspension and cells were pelleted by centrifugation (200g for 5 min), resuspended in culture media and seeded into new flasks.

Human iPSC lines were cultured on Geltrex (ThermoFisher) coated

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 Panels A, B
Phenotype	Qualitative analysis: Immunocytochemistry	Positive for the pluripotency markers: OCT4, NANOG, SOX2	Fig. 1 Panels E, F
	Quantitative analysis: RT-qPCR	Positive for the pluripotency markers: *OCT4, NANOG, SOX2 and KLF4*	Fig. 1 Panel G
Genotype	Illumina Beadchip SNP Array with CNV Analysis	46XY 500 kbp resolution	Fig. 1 Panels C, D
Identity	STR analysis	DNA profiling performed Matched at 16 loci	Archived with the journal
Mutation analysis	Sequencing	Compound heterozygous for *ABCA4* c.768G > T and c.6079C > T	Fig. 1 Panels A, B
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma – Lookout Mycoplasma PCR Detection Kit	Mycoplasma testing by PCR: Negative	Supplementary Fig. S1A
Differentiation potential	e.g. Embryoid body formation: qPCR	Positive for trilineage markers *PAX6, OTX1, IGF2, BMP4, GATA4*	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



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culture plates in TeSR-E8 medium (StemCell Technologies) and were passaged every 4–5 days using a split ratio of 1:3–1:5. For passaging, culture media was removed and replaced with EDTA solution (0.5 mM EDTA in PBS) and incubated at room temperature for 4–5 min. EDTA was then removed and replaced with StemFlex media and iPSC colonies dissociated into floating cell clusters by gentle scraping with a bent 200 µl tip. The resulting suspension was then seeded onto new Geltrex coated 6 well plates. Both fibroblasts and iPSC cultures were cultured at 37 °C with 5% CO2. A commercial iPSCline (ThermoFisher, Cat#A18945, HuiPSC) was used as a control.

Fig. 1. A–B) LEIi012-A (A) and LEIi012-B (B) displayed typical iPSC colony morphology in culture. Targeted Sanger sequencing demonstrated the presence of compound heterozygous mutations (c.768G > T and c.6079C > T) in the *ABCA4* gene in both iPSC lines. **C–D**) Digital karyotyping was performed on the LEIi012-A (C) and LEIi012-B (D) iPSC lines using the Infinium HumanCytoSNP-12 Beadchip SNP array, followed by copy number variation analysis. B-allele frequencies (BAF, upper panels) and LogR values (lower panels) were plotted against genomic location for 300,000 human SNPs, demonstrating both lines had normal 46, XY karyotypes. **E–F**) Immunostaining analysis demonstrated expression of the pluripotency markers OCT4, NANOG, SOX2 and TRA-1-81 in LEIi012-A (E) and LEIi012-B (F) iPSC (upper panels). Nuclei were counter stained with DAPI (merged lower panels). **G**) Quantitative RT-PCR analysis demonstrated *OCT4*, *NANOG*, *SOX2* and *KLF4* were expressed at similar levels in LEIi012-A (light grey bars), LEIi012-B (dark grey bars) and a control human iPSC line (black bars, HuiPS). Expression of each marker was normalized to *GAPDH* expression. Each bar shows the mean value obtained for iPSC lines across three different passages. Error bars indicate standard deviation. **H**) Embryoid bodies derived from LEIi012-A (light grey bars) and LEIi012-B (dark grey bars) iPSC showed upregulation of endoderm (*AFP*), mesoderm (*GATA2*, *IGF2*) and ectoderm (*PAX6, OTX1*) markers, and downregulation of *OCT4* expression, compared with undifferentiated iPSC. Expression of each marker was normalized to *GAPDH* expression. Each bar shows the mean value obtained for iPSC lines across three different passages or from three independent embryoid body cultures for each line. Error bars indicate the standard deviation.

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Mouse anti-OCT4	1:200	Stem Cell Technologies Cat# 60093.1, RRID: AB_2561766
	Rabbit anti-NANOG	1:100	Abcam Cat# ab21624. RRID: AB_446437
	Rabbit anti-SOX2	1:200	ThermoFisher Scientific Cat# 48-1400, RRID: AB_2533841
Secondary antibodies	Goat anti-mouse Alexa Fluor 546	1:500	Molecular Probes Cat# A-11003, RRID:AB_141370
	Goat anti-rabbit-Alexa Fluor 488	1:500	Molecular Probes Cat# A-11008, RRID: AB_143165
Primers			
	Target	Forward/Reverse primer (5′-3′)	
Episomal Plasmid (PCR)	Reprogramming plasmids	AGGTCCCTCGAAGAGGTTCA/TTCCAACGCGAGAAGGTGTT	
Pluripotency Markers (qPCR)	*OCT4*	CCTGAAGCAGAAGAGGATCACC/AAAGCGGCAGATGGTCGTTTGG	
	NANOG	CTCCAACATCCTGAACCTCAGC/CGTCACACCATTGCTATTCTTCG	
	SOX2	GCTACAGCATGATGCAGGACCA/TCTGCGAGCTGGTCATGGAGTT	
	KLF4	CATCTCAAGGCACACCTGCGAA/TCGGTCGCATTTTTGGCACTGG	
House-Keeping Genes (qPCR)	*GAPDH*	GTCTCCTCTGACTTCAACAGCG/ACCACCCTGTTGCTGTAGCCAA	
Trilineage markers (qPCR)	*PAX6*	CTGAGGAATCAGAGAAGACAGGC/ATGGAGCCAGATGTGAAGGAGG	
	OTX1	CTACCCTGACATCTTCATGCGG/GGAGGGACTTCTTCTTGGCTG	
	IGF2	AGACGTACTGTGCTACCCC/TGCTTCCAGGTGTCATATTGG	
	AFP	TGAGCACTGTTGCAGAGGAG/TTGTTTGACAGAGTGTCTTGTTGA	
	GATA2	CTGTCTGCAACGCCTGTG/GTTCCGAGTCTGGATCCCTT	
Mutation sequencing	*ABCA4* c.768G > T	CCTCTTCTCCCTGCAGTTCG/GACACAAACATGCCACAGCC	
	ABCA4 c.6079C > T	GCAGCTTCCTTTTCCCGTTG/ATTTTGCACTGTGGGAGATGC	

4.3. Reprogramming

Patient fibroblasts were reprogrammed using the Episomal iPSC Reprogramming Plasmid kit (SC900A-1, System Biosciences), according to the manufacturer's instructions. 1 million patient fibroblast cells were electroporated using the NEON electroporation system (3 × 10 ms pulses at 1650 V in a 100 µl tip), plated into 6 wells of a 6 well plate containing DMEM medium supplemented with 10% fetal calf serum and cultured for 7 days. On day 7, the media was changed to TeSR-E8. On day 25, individual iPSC colonies were picked for clonal expansion.

4.4. Trilineage differentiation

To promote trilineage differentiation, iPSCs were spontaneously differentiated as embryoid bodies over 14 days. On day 1, iPSC cluster suspensions were prepared as described above for passaging and seeded into 6 well suspension culture plates containing mTesR1 media and 10 µM Y27632 (Abcam). On day 3 of culture, media was changed to DMEM/F12 supplemented with 20% knockout serum replacement, antibiotic–antimycotic (ThermoFisher) and MEM non-essential amino acid solution (StemCell Technologies). Media was changed daily, and after 14 days RNA was harvested for qPCR analysis.

4.5. Quantitative PCR analysis

Total RNA was isolated from cell culture samples using TRIzol reagent. cDNA was synthesized using the RT^2 First Strand Kit (Qiagen). qPCR was performed using the CFX Connect Real-Time System (BioRad) with the PowerSYBR Green PCR Master Mix (Applied Biosystems) and data analyzed using the ΔC_T method. Gene expression values were normalized to the expression of the *GAPDH* housekeeping gene (Fig. 1G-H). For each line, mean expression values and standard deviations were calculated from three independently cultured iPSC or EB cDNA samples. Primers used in qPCR assays are listed in Table 3.

4.6. Immunostaining analysis

iPSCs were cultured on Geltrex-coated coverslips for 5 days before being fixed with 4% paraformaldehyde. Fixed iPSCs were washed with phosphate buffered saline (PBS), and permeabilized using PBS with 0.3% Triton X-100 for 15 min. iPSCs were incubated for 1 h at room temperature in blocking buffer (PBS containing 0.3% Triton X-100 added to normal goat serum at 5% final concentration). Primary antibodies were diluted in blocking buffer and applied at 4°C overnight, followed by three washes with PBS. Secondary antibodies and DAPI (1 µg/mL) stains were diluted in blocking buffer and applied for 2 h at 4°C. The antibodies used and their respective dilutions are listed in Table 3. Coverslips were then washed three times in PBS and mounted onto slides. Stained cells were visualized using the Olympus BX60 fluorescence microscope equipped with DP-Controller 3.1.1.267 acquisition software (Olympus Corporation, Tokyo, Japan) and merged using ImageJ software (version 1.43).

4.7. Digital karyotyping and microsatellite analysis

To identify genomic insertions or deletions, digital karyotyping was performed on LEIi012-A and LEIi012-B at passage 11 using the Infinium

HumanCytoSNP-12 Beadchip SNP array (Illumina, Australian Genome Research Facility). Balanced translocations are not detected using this method. CNV analysis was performed using GenomeStudio2.0 software with the CNVpartition 2.0 plugin (Illumina). Microsatellite analysis was performed using the Promega Powerplex 16H system (Australian Genome Research Facility).

4.8. Mycoplasma and episomal testing

The Lookout Mycoplasma PCR Detection Kit (Sigma-Aldrich) was used for mycoplasma detection according to the manufacturer's recommendations. Episomal PCR analysis used primers to detect episomal plasmids within LEIi012-A and LEIi012-B (primer target sequences listed in Table 3).

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2020.101947.

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