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Lab Resource: Multiple Cell Lines

# Generation of four iPSC lines from four patients with Leigh syndrome carrying homoplasmic mutations m.8993T > G or m.8993T > C in the mitochondrial gene *MT-ATP6*

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A B S T R A C T

We report the generation of four human iPSC lines (8993-A12, 8993-B12, 8993-C11, and 8993-D7) from fibroblasts of four patients affected by maternally inherited Leigh syndrome (MILS) carrying homoplasmic mutations m.8993T > G or m.8993T > C in the mitochondrial gene *MT-ATP6*. We used Sendai viruses to deliver reprogramming factors OCT4, SOX2, KLF4, and c-MYC. The established iPSC lines expressed pluripotency markers, exhibited a normal karyotype, were capable to form cells of the three germ layers *in vitro*, and retained the *MT-ATP6* mutations at the same homoplasmic level of the parental fibroblasts.

## Resource table

| | |
|---|---|
| Unique stem cell lines identifier | MDCi007-A<br>MDCi008-A<br>MDCi009-A<br>MDCi010-A |
| Alternative names of stem cell lines | 8993-A12 (MDCi007-A)<br>8993-B12 (MDCi008-A)<br>8993-C11 (MDCi009-A)<br>8993-D7 (MDCi10-A) |
| Institution | Max Delbrueck Center for Molecular Medicine (MDC), Berlin, Germany; Heinrich Heine University, Düsseldorf, Germany |
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| Type of cell lines | iPSCs |
| Origin | Human |
| Cell Source | Human dermal fibroblasts cells |

(*continued on next column*)

**Resource table** (*continued*)

| | |
|---|---|
| Clonality | Clonal |
| Method of reprogramming | Transgene free (CytoTune-iPS 2.0 Sendai Reprogramming Kit, Thermo Fisher Scientific) |
| Multiline rationale | Same disease non-isogenic cell lines |
| Gene modification | yes |
| Type of modification | Maternally inherited |
| Associated disease | Maternally inherited Leigh syndrome (MILS) |
| Gene/locus | Mutations in the mitochondrial DNA gene *MT-ATP6*: mutation m.8993T > G: iPSC lines 8993-A12, 8993-C11, and 8993-D7<br>mutation m.8993T > C: iPSC line 8993-B12 |
| Method of modification | N/A |
| Name of transgene or resistance | N/A |
| Inducible/constitutive system | N/A |
| Date archived/stock date | N/A |
| Cell line repository/bank | https://hpscreg.eu/cell-line/MDCi007-A<br>https://hpscreg.eu/cell-line/MDCi008-A<br>https://hpscreg.eu/cell-line/MDCi009-A<br>https://hpscreg.eu/cell-line/MDCi010-A |

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**Resource table** (*continued*)

| | |
|---|---|
| Ethical approval | The original study was approved by the IRB of the Charité (EA2/131/13 and EA2/107/14). The work was then approved by the Ethic Committee of the Medical Faculty of Heinrich Heine University (study number: 2020-967_2).<br>The lines cannot be freely shared under the current ethical approval. In case of interest, in addition to an appropriate MTA, a modified ethical approval and updated patient consent forms will be required. |

## 1. Resource utility

There is a lack of animal and cellular models for mitochondrial DNA (mtDNA)- associated Leigh syndrome (MILS) because of the difficulty to engineer mtDNA. Patient-derived iPSCs allow the development of effective cellular models of MILS to study disease mechanisms and perform drug discovery (Lorenz et al., 2017).

## 2. Resource details

Leigh syndrome (OMIM #256000) is an incurable neurodevelopmental disorder and the most severe pediatric manifestation of mitochondrial disease (Baertling et al., 2014). mtDNA-associated Leigh syndrome also known as maternally inherited Leigh syndrome (MILS) is typically caused by mutations in the mtDNA gene *MT-ATP6* encoding for a subunit of the ATP synthase, complex V of the mitochondrial respiratory chain (Ganetzky et al., 2019). Cells contain numerous mtDNA copies. A high percentage of copies must be mutated before clinical MILS symptoms occur. Hence, either the great majority of mtDNA copies are mutated (heteroplasmy), or virtually all mtDNA copies are mutated (homoplasmy).

We obtained somatic skin fibroblasts from four individuals affected by MILS: patient A (male, 2 months old), patient B (male, 9 years old), patient C (female, 2 years old), and patient D (male, 3 years old) **(**Table 1**)**. Three patients carried the *MT-ATP6* mutation m.8993T > G (patient A, C, and D). This is the most frequent mutation associated with MILS (Ganetzky et al., 2019; Holt et al., 1990). One patient (patient B) carried the mutation m.8993T > C, which is also linked to MILS (de Vries et al., 1993; Ganetzky et al., 2019). All patient fibroblasts carried the mutations at homoplasmic level.

We used non-integrative Sendai viruses containing the reprogramming factors OCT3/4, SOX2, c-MYC, and KLF4 to generate induced pluripotent stem cells (iPSCs) (Table 2). From each fibroblast, we generated one iPSC line: 8993-A12 from patient A, 8993-B12 from patient B, 8993-C11 from patient C, and 8993-D7 from patient D (Table 2). The four iPSC lines showed a typical human embryonic stem cell-like colony morphology and growth behaviour, and expressed pluripotency-associated protein markers OCT4, NANOG, and TRA-1–60 at passage 16 (Fig. 1**A** scale bars 200 μm for colonies, and 50 μm for others). Clearance of vectors and exogenous reprogramming factor genes was confirmed by RT-PCR after nine culture passages **(Fig. S1A)**. The endogenous expression of the pluripotency-associated transcription factors NANOG, SOX2, DNMT3B, and DPPA4 was confirmed by RT-qPCR at passage 20 and compared to healthy control iPSC line TFBJ (Lorenz et al., 2017) **(**Fig. 1**B)**. The presence of mtDNA mutations in the *MT-ATP6* gene was monitored using PCR–restriction fragment length polymorphism (PCR-RFLP) analyses. Similar to parental fibroblasts, all iPSCs contained the *MT-ATP6* mutations at homoplasmic level at passage 20 **(**Fig. 1**C)**. Using *in vitro* embryoid body (EB)-based differentiation, we confirmed the capacity of the four iPSC lines to give rise to cells belonging to the three germ layers showing the expression of protein markers indicative of mesoderm (alpha-smooth muscle actin SMA and fibronectin FN), endoderm (alpha-fetoprotein AFP and SOX17), and ectoderm (PAX6 and TUJ1) (Fig. 1**D**, scale bars 100 μm). The four iPSC lines (8993-A12, 8993-B12, 8993-C11, and 8993-D7) have been adapted to feeder-free culture conditions and displayed normal karyotypes at passage 30 **(**Fig. 1**E)**. STR analysis confirmed that the iPSC lines were derived from the relative patient fibroblasts.

**Table 2**
Characterization and validation.

| Classification | Test | Result | Data |
|---|---|---|---|
| **Morphology** | Microscopy | Normal | Fig. 1A |
| **Phenotype** | Qualitative analysis: Immunocytochemistry | Positive for OCT4, NANOG, TRA-1–60 | Fig. 1A |
| | Quantitative analysis: RT-qPCR | Positive for OCT4, NANOG, SOX2, GDF3, DPPA4, DNMT3B | Fig. 1B |
| **Genotype** | SNP array | *46XY, 46XY, 46XX, 46XY* Resolution: 0.5 megabases | Fig. 1E |
| **Identity** | STR analysis andmicrosatellite PCR (mPCR) | STR analysis | Not shown but available with author |
| **Mutation analysis (IF APPLICABLE)** | PCR–restriction fragment length polymorphism (PCR-RFLP) | All iPSC lines contained *MT-ATP6* mutations at homoplasmic level | Fig. 1C |
| | Southern Blot OR WGS | N/A | |
| **Microbiology and virology** | Mycoplasma | Mycoplasma testing by PCR: Negative | Fig. S1B |
| **Differentiation potential** | Embryoid body formation | Immunostaining positive for SMA and fibronectin (mesoderm), AFP and SOX17 (endoderm), and PAX6 and TUJ1 (ectoderm) | Fig. 1D |
| **Donor screening (OPTIONAL)** | HIV 1 + 2 Hepatitis B, Hepatitis C | Negative | Not shown but available with author |
| **Genotype additional info (OPTIONAL)** | Blood group genotyping | N/A | N/A |
| | HLA tissue typing | N/A | N/A |

**Table 1**
Summary of lines.

| iPSC line names | Abbreviation in figures | Gender | Age | Ethnicity | Genotype of locus | Disease |
|---|---|---|---|---|---|---|
| 8993-A12 | iPSC_8993-A12, iPSC_A12 | Male | 2 months | Caucasian | *MT-ATP6* m.8993T > G | Leigh syndrome |
| 8993-B12 | iPSC_8993-B12, iPSC_B12 | Male | 9 years | Caucasian | *MT-ATP6* m.8993T > C | Leigh syndrome |
| 8993-C11 | iPSC_8993-C11, iPSC_C11 | Female | 2 years | Caucasian | *MT-ATP6* m.8993T > G | Leigh syndrome |
| 8993-D7 | iPSC_8993-D7, iPSC_D7 | Male | 3 years | Caucasian | *MT-ATP6* m.8993T > G | Leigh syndrome |

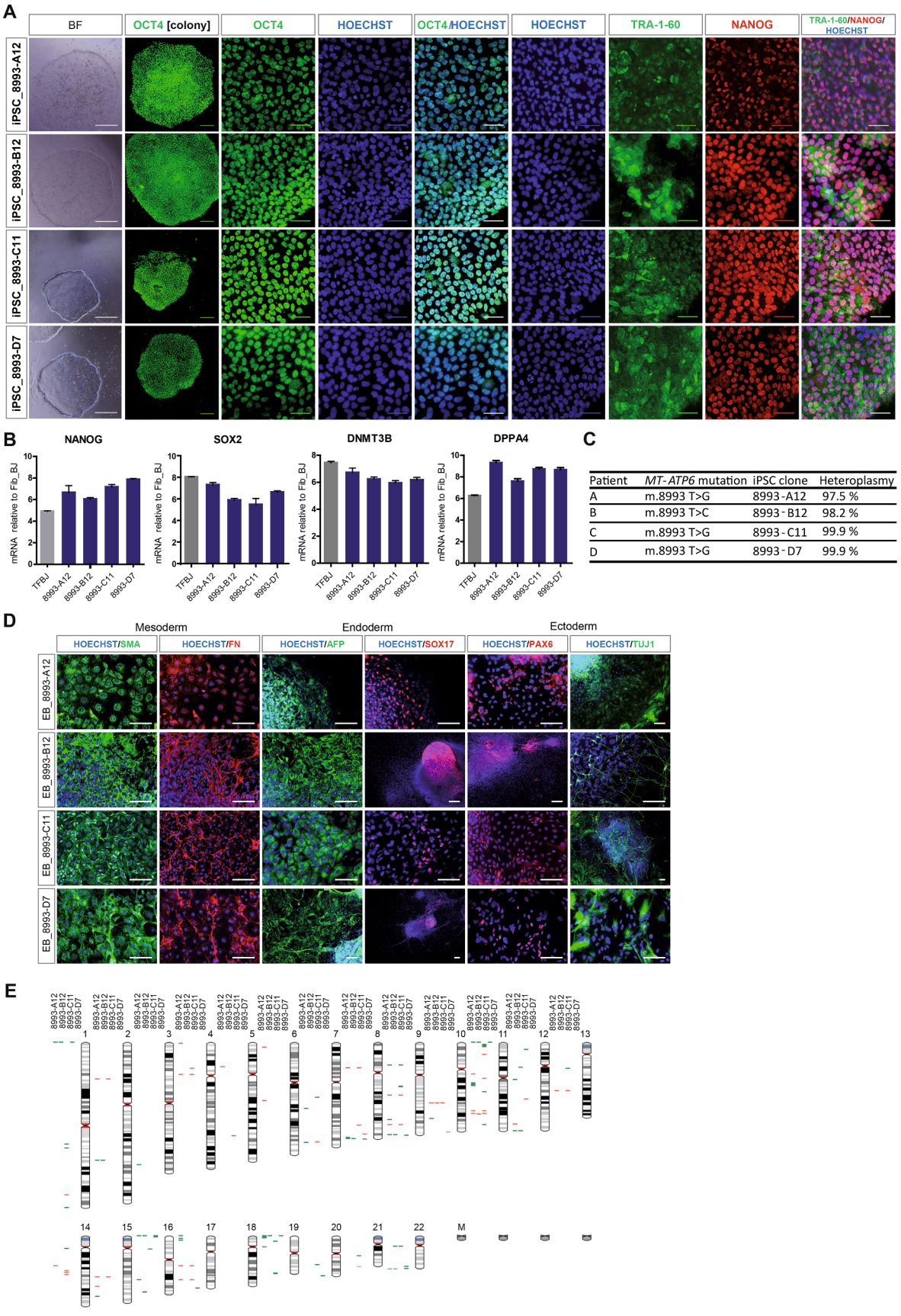


**Fig. 1.** The four iPSC lines (8993-A12, 8993-B12, 8993-C11, and 8993-D7) expressed pluripotency-associated markers, differentiated into the three germ layers, were karyotypically normal, and carried homoplasmic *MT-ATP6* mutations.

**Table 3**
Reagents details.

| **Antibodies used for immunocytochemistry/flow-citometry** | | | |
|---|---|---|---|
| | **Antibody** | **Dilution** | **Company Cat # and RRID** |
| Pluripotency Marker | Mouse monoclonal anti-OCT-3/4 (C-10) | 1:300 | Santa Cruz Cat# sc-5279 |
| Pluripotency Marker | Mouse monoclonal anti-TRA-1–60 | 1:200 | Millipore Cat# MAB4360 |
| Pluripotency Marker | Goat polyclonal anti-NANOG | 1:200 | R&D Systems Cat# AF1997 |
| Differentiation Marker | Mouse monoclonal anti-SMA, clone 1A4 | 1:200 | DakoCytomation Cat# M0851 |
| Differentiation Marker | Rabbit polyclonal anti-PAX6 | 1:200 | BioLegend Cat# 901301 |
| Differentiation Marker | Rabbit polyclonal anti-Fibronectin | 1:300 | Sigma Cat# F3648 |
| Differentiation Marker | Mouse monoclonal anti-AFP (1G7) | 1:100 | Sigma Cat# WH0000174M1 |
| Differentiation Marker | Goat polyclonal anti-SOX17 | 1:50 | R&D Systems Cat# AF1924 |
| Differentiation Marker | Mouse monoclonal anti-ß-Tubulin III (TUJ1) | 1:2000 | Sigma Cat# T8578 |
| Secondary antibody | Cy5-conjugated AffiniPure Goat anti-Rabbit IgG (H + L) | 1:300 | Jackson Immuno Research Cat# 111–175-144 |
| Secondary antibody | Cy3-conjugated Donkey anti-Mouse | 1:300 | Merck Millipore Cat# AP192C |
| Secondary antibody | Cy5-conjugated AffiniPure Donkey anti-Goat IgG (H + L) | 1:300 | Jackson Immuno Research Cat# 705–175-147 |
| **Primers** | | | |
| | **Target** | **Forward/Reverse primer (5′-3′)** | |
| Pluripotency Markers (qPCR) | NANOG | F: CCTGTGATTTGTGGGCCTG and R: GACAGTCTCCGTGTGAGGCAT | |
| Pluripotency Markers (qPCR) | SOX2 | F: GTATCAGGAGTTGTCAAGGCAGAG and R: TCCTAGTCTTAAAGAGGCAGCAAAC | |
| Pluripotency Markers (qPCR) | DPPA4 | F: TGGTGTCAGGTGGTGTGTGG and R: CCAGGCTTGACCAGCATGAA | |
| Pluripotency Markers (qPCR) | DNMT3B | F: GCTCACAGGGCCCGATACTT and R: GCAGTCCTGCAGCTCGAGTTTA | |
| House-Keeping Genes (qPCR) | ACTB | F: TCAAGATCATTGCTCCTCCTGAG and R: ACATCTGCTGGAAGGTGGACA | |
| House-Keeping Genes (qPCR) | GAPDH | F: CTGGTAAAGTGGATATTGTTGCCAT and R: TGGAATCATATTGGAACATGTAAACC | |
| Sendai virus genome detection (RT-PCR) | SeV | F: GGATCACTAGGTGATATCGAGC and R: ACCAGACAAGAGTTTAAGAGATATGTATC | |
| Transgene detection (RT-PCR) | KOS | F: ATGCACCGCTACGACGTGAGCGC and R: ACCTTGACAATCCTGATGTGG | |
| Transgene detection (RT-PCR) | Klf4 | F: TTCCTGCATGCCAGAGGAGCCC and R: AATGTATCGAAGGTGCTCAA | |
| Transgene detection (RT-PCR) | c-Myc | F: TAACTGACTAGCAGGCTTGTCG and R: TCCACATAC AGTCCTGGATGATGATG | |
| Mycoplasma test | Myco-f1 | F: CGCCTGAGTAGTACGTTCGC | |
| Mycoplasma test | Myco-f2 | F: CGCCTGAGTAGTACGTACGC | |
| Mycoplasma test | Myco-f3 | F: TGCCTGAGTAGTCACTTCGC | |
| Mycoplasma test | Myco-f4 | F: CGCCTGGGTAGTACATTCGC | |
| Mycoplasma test | Myco-f5 | F: CGCCTGAGTAGTAGTCTCGC | |
| Mycoplasma test | Myco-f6 | F: TGCCTGGGTAGTACATTCGC | |
| Mycoplasma test | Myco-r1 | R: GCGGTGTGTACAAGACCCGA | |
| Mycoplasma test | Myco-r2 | R: GCGGTGTGTACAAAACCCGA | |
| Mycoplasma test | Myco-r3 | R: GCGGTGTGTACAAACCCCGA | |
| mtDNA mutation analysis | m.8993T > G/C | F: AGCCTACTCATTCAACCAATAGCCC | |
| mtDNA mutation analysis | m.8993T > G/C | R: FAM-GGCGACAGCGATTTCTAGGA | |

## 3. Materials and methods

### 3.1. iPSC reprogramming

Human fibroblasts were reprogrammed using CytoTune-iPS 2.0 Sendai kit (Thermo Fisher). Silencing of exogenous factor genes and Sendai virus genome was confirmed by RT-PCR (**Fig. S1A**). All iPSC lines were maintained in feeder-free conditions with StemMACS iPS-Brew XF (Miltenyi Biotec) and MycoZap. iPSCs were kept in humidified atmosphere of 5% $CO_2$ at 37 °C and 5% oxygen. Pluripotency was confirmed using embryoid bodies (EBs) grown in suspension for 1 week and adherent for 10 days using KO-DMEM medium (GIBCO), 20% knock-out serum replacement (GIBCO), MycoZap, non-essential amino acids, and Pen/Strep (Lorenz et al., 2017). For detection of mycoplasmal DNA, PCR analysis of supernatant from cell culture (**Fig. S1B**) was performed using a set of primers (Table 3).

### 3.2. Immunostaining

Cells grown on Matrigel-coated coverslips were fixed with 4% paraformaldehyde (Science Services) for 20 min at room temperature (RT) and washed three times with PBS. Cells were incubated with blocking solution containing 10% normal donkey serum (Abcam) and 1% Triton X-100 (Sigma-Aldrich) in PBS with 0.05% Tween 20 (Sigma-Aldrich) for 1 hr at RT. Primary antibodies (Table 3) were incubated overnight at 4 °C. Cells were then washed three times and incubated with secondary antibodies (Table 3) for 1 hr at RT. Nuclei were counterstained with 1:10,000 Hoechst (Thermo Fisher). Images were acquired with LSM510 Meta (Zeiss) and AxioVision V4.6.3.0 software (Zeiss), and processed with AxioVision software and ImageJ.

### 3.3. RT-qPCR

Total RNA was isolated using RNeasy Mini Kit (QIAGEN, Valencia, CA). cDNA samples were measured in triplicates using 384-Well Optical Reaction Plates (Applied Biosystems). Gene expression analysis was performed with a set of primers (Table 3) using SYBR Green PCR Master Mix and ViiA 7 Real-Time PCR (Applied Biosystems), using the $2^{-\Delta\Delta CT}$ method. Data were normalized to the housekeeping genes ACTB and GAPDH and presented as mean log2 ratios in relation to the control fibroblasts BJ (from ATCC) from which the control iPSC line TFBJ was previously derived (Lorenz et al., 2017).

### 3.4. STR analysis

STR analysis was performend at the Institut für Rechtsmedizin at Universitätsklinikum Düsseldorf (UKD). DNA was isolated with FlexiGene DNA Kit (QIAGEN). 21 microsatellite loci were amplified via PCR

and labelled products were analysed with GeneMapper ID v.3.2.1 (Applied Biosystems).

### 3.5. mtDNA mutation analysis

Total genomic DNA was isolated from patient fibroblasts and iPSCs using FlexiGene DNA kit (QIAGEN).

To quantify the level of *MT-ATP6* mutations in iPSCs, we carried out PCR–restriction fragment length polymorphism (PCR-RFLP) analyses with restriction enzyme *HpaII* (10,000 units/ml). In wild-type samples without *MT-ATP6* mutations, the restriction enzyme cuts the products into two fragments (25 bp + 155 bp). In samples containing *MT-ATP6* mutations, the products remain uncut (180 bp). The percentage of mutation was quantified by real-time PCR with a set of primers (Table 3).

### 3.6. Karyotyping

DNA was isolated using the DNeasy blood and tissue kit (QIAGEN). SNP karyotyping was assessed using the Infinium OmniExpressExome-8 Kit and the iScan system from Illumina. CNV and SNP visualization were performed using KaryoStudio v1.4 (Illumina).

## 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

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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.2022.102742.

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