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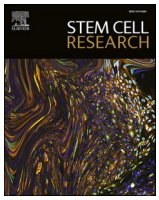

Lab Resource: Multiple Cell Lines

Generation of two mother–child pairs of iPSCs from maternally inherited Leigh syndrome patients with m.8993 T > G and m.9176 T > G *MT-ATP6* mutations

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A B S T R A C T

We generated two pairs of mother–child iPSCs lines for Maternally Inherited Leigh Syndrome (MILS) carrying the m.8993 T > G and m.9176 T > G mutations in the *MT-ATP6* gene. We delivered reprogramming factors OCT4, SOX2, KLF4, and c-MYC *via* Sendai virus. All iPSCs lines had a normal karyotype, expressed pluripotency markers, and differentiated into the three germ layers. Both patient-iPSCs retained the same degrees of heteroplasmy as their source fibroblasts (>97.0 %). In maternal iPSCs, the heteroplasmy remained 0.0 % in the case of the m.8993 T > G mutation and dropped from 55.0 % to 1.0 % in the case of m.9176 T > G mutation.

1. Resource Table

Unique stem cell lines identifier	BIHi267-B BIHi276-A BIHi269-B BIHi266-A
Alternative name(s) of stem cell lines	N/A
Institution	Berlin Institute of Health (BIH)
Contact information of distributor	Markus Schuelke, MD markus.schuelke@charite.de
Type of cell lines	iPSCs
Origin	Human
Additional origin info required	BIHi267-BBIHi276-ABIHi269-BBIHi266-A Age:1714231 Sex:MaleMaleFemaleFemale
Cell Source	Skin fibroblasts
Clonality	Clonal
Method of reprogramming	Episomally using Sendai virus
Genetic modification	No
Type of genetic modification	Wildtype cells derived iPSCs with a naturally occurring mtDNA mutation, no genetic modification done
Evidence of the reprogramming transgene loss	RT-qPCR
Associated disease	Maternally Inherited Leigh Syndrome (MILS)
Gene/locus	BIHi267-B and BIHi269-B: mutation m.9176 T > G in *MT-ATP6* BIHi276-A and BIHi266-A: mutation m.8993 T > G in *MT-ATP6*
Date archived/stock date	BIHi267-B: 17.03.2020 BIHi276-A: 19.09.2020 BIHi269-B: 07.06.2020 BIHi266-A: 06.03.2020
Cell line repository/bank	https://hpscreg.eu/cell-line/BIHi266-A https://hpscreg.eu/cell-line/BIHi267-B https://hpscreg.eu/cell-line/BIHi269-B https://hpscreg.eu/cell-line/BIHi276-A
Ethical approval	Ethikkommission Ethikausschuss am Campus Virchow-Klinikum; EA2/131/13. The lines cannot

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	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.

2. Resource utility

In the absence of transgenic technologies to manipulate the mitochondrial DNA (mtDNA), the generation of a disease model for Maternally Inherited Leigh Syndrome (MILS) has been challenging in the past. The cell lines described here represent a MILS disease model suitable for various cell-based studies based on two common mutations. Further, both patient cell lines are complemented with the maternally derived iPSC lines that can serve as isogenic controls with regard to the mtDNA (See Table 1).

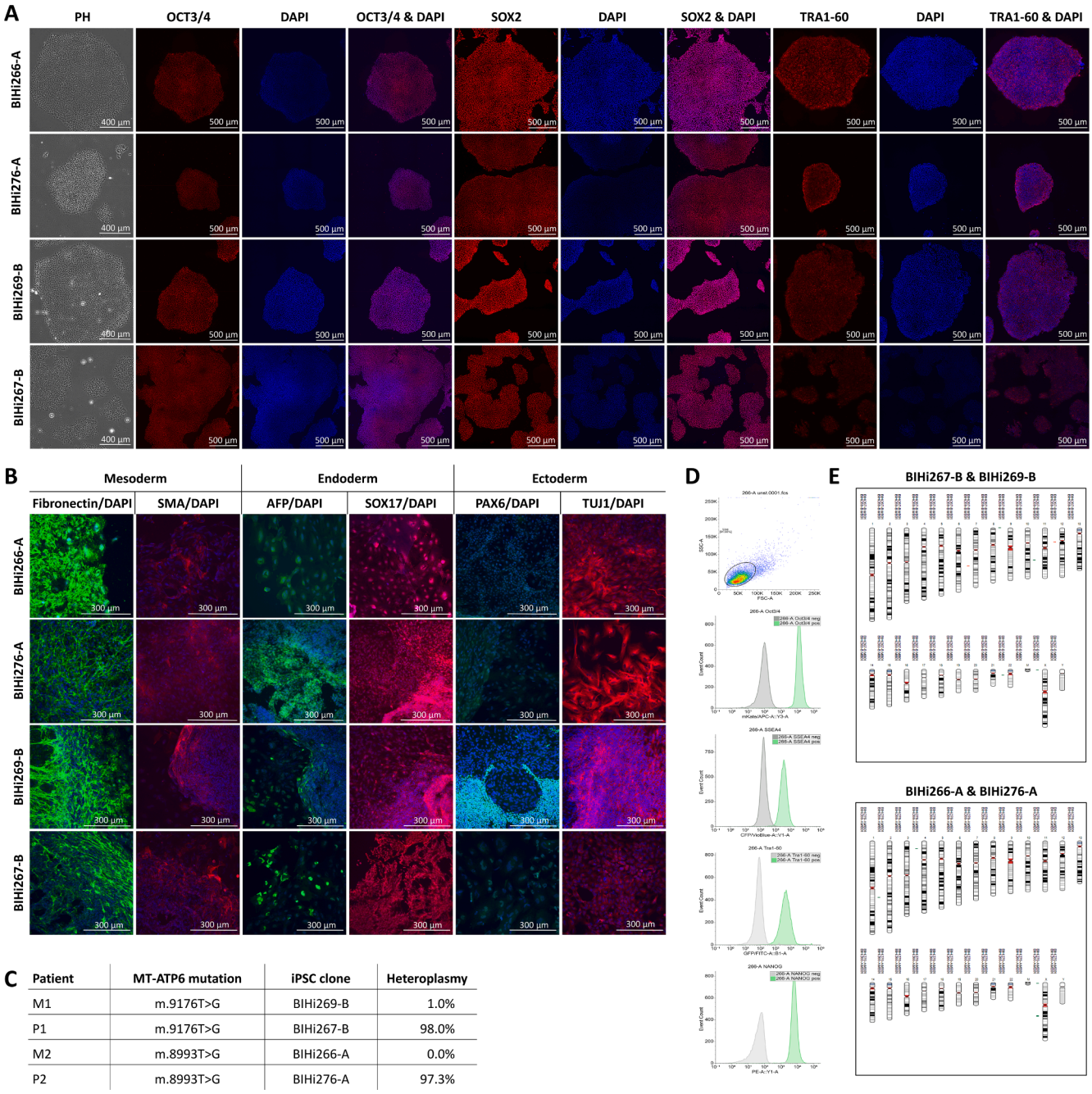
3. Resource details

Leigh Syndrome (LS, OMIM #256000) was first described in 1951 by the physician Denis Leigh as "Subacute necrotizing encephalomyelopathy" (Leigh, 1951). It is a rare neurodevelopmental mitochondrial disease. LS often manifests during early infancy after a seemingly normal developmental period. Manifesting symptoms such as muscle weakness, brain stem symptoms, cranial nerve palsies, epilepsy, or dystonia may be triggered by febrile illness or catabolic states (Lim et al., 2022). Presently there is no pathogenicity based curative therapy for LS. The disease is caused by mutations in the nuclear or mitochondrial genome that affect structural proteins and assembly factors of respiratory chain components leading to an overall decreased intracellular ATP production. Neurons and muscle cells are thus most often affected by these biochemical abnormalities leading to necrosis in the basal ganglia and to muscle weakness. The most frequent LS-related mtDNA mutations are located in the *MT-ATP6* gene, which encodes a subunit of the respiratory chain complex V (F_0F_1-ATPase). This ATPase6-subunit is localized in subcomplex F_0 and carries the proton pore, a crucial component for the function of the holocomplex. Each cell carries from hundreds to thousands of mitochondria, each in turn populated by 2–10 mtDNA copies. These mtDNA copies might either all harbour the mutation, a state called "homoplasmy" or only a portion, called "heteroplasmy". The degree of heteroplasmy often defines disease manifestation and severity. Due to deranged calcium signalling (Lorenz et al., 2017), the most frequently affected neurons in the brain are dopaminergic neurons in brainstem and basal ganglia, which tend to degenerate in a characteristic distribution.

The here described iPSCs were reprogrammed from skin fibroblasts of two MILS patients and their mothers: patient A (P1, BIHi267-B, m.9176 T > G, male, 17 years) and his mother (M1, BIHi269-B, female, 42 years), patient B (P2, BIHi276-A, m.8993 T > G, male, 1 year) and his mother (M2, BIHi266-A, female, 31). Both patient source fibroblasts were nearly homoplasmic for the respective *MT-ATP6* mutation. Induced pluripotent stem cells (iPSCs) were produced by transduction of each skin fibroblast line with non-integrating Sendai viruses carrying the reprogramming factors OCT3/4, SOX2, c-MYC, and KLF4. We confirmed the clearing of vectors and of exogenous reprogramming factors by RT-qPCR at passage #10 (BIHi276-A), #12 (BIHi269-B), and #13 (BIHi266-A, BIHi267-B) (Supplementary files). Light microscopic analysis showed a typical human iPSC-like colony morphology and growth behaviour under feeder-free and hypoxic conditions (Fig. 1A, scale bars 400 μm). Immunofluorescence (IF) staining confirmed expression of pluripotency-associated protein markers (OCT4, SOX2, TRA-1–60) at passage #16 (BIHi269-B, BIHi276-A), #20 (BIHi267-B), and #21 (BIHi266-A) (Fig. 1A, scale bars 500 μm). Further, endogenous expression of pluripotency-associated transcription factors

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography bright field	Typical morphology of undifferentiated hPSCs	Fig. 1A
Phenotype	Qualitative analysis	Staining/expression of pluripotency markers: OCT3/4, SOX2, TRA1-60	Fig. 1A
	Quantitative analysis	Expression of at least three pluripotency markers detected BIHi267-BBIHi276-ABIHi269-BBIHi266-A OCT3/499.6 %99.9 %99.2 %99.8 % TRA1-6094.9 % 96.6 %97.9 %99.4 % NANOG99.6 %99.9 %99.5 %99.8 % SSEA497.9 %96.8 % 95.7 %99.0 % conclusionover 80 % positiveover 80 % positiveover 80 % positive	Fig. 1D and Supplementary material
Genotype	Karyotype (G-banding) and resolution	Illumina, OMNI-EXPRESS-8v1.4 Chip; No significant changes compared to the primary cells detected	Fig. 1E
Identity	Microsatellite PCR (mPCR) OR STR analysis	N/A Detection of nine human loci; identical to profile of primary cells	Submitted in archive with journal
Mutation analysis	Sequencing	BIHi267-BBIHi276-ABIHi269-BBIHi266-A m.9176 T > G 98.0 %m.8993 T > G 97.3 %m.9176 T > G 1.0 %m.8993 T > G 0 %	Fig. 1B
	Southern blot OR WGS	N/A	
Microbiology and virology	Mycoplasma	qPCR-based Venor®*GeM* qOneStep Kit negative	Supplementary material
Differentiation potential	Embryoid body formation OR teratoma formation OR scorecard OR directed differentiation	Proof of three germ layers formation.	Fig. 1C
List of recommended germ layer markers	Expression of these markers has been demonstrated at protein (IF) levels, at least 2 markers need to be shown per germ layer	Ectoderm: PAX6, TUBB3/TUJ1 Endoderm: SOX17 Mesoderm: Fibronectin FN, SMA	Fig. 1C
Donor screening	HIV 1 + 2 Hepatitis B, Hepatitis C	negative	Not shown but available with author
Genotype additional info	Blood group genotyping	N/A	
	HLA tissue typing	N/A	



C

Patient	MT-ATP6 mutation	iPSC clone	Heteroplasmy
M1	m.9176T>G	BIHi269-B	1.0%
P1	m.9176T>G	BIHi267-B	98.0%
M2	m.8993T>G	BIHi266-A	0.0%
P2	m.8993T>G	BIHi276-A	97.3%

Fig. 1. Derivation and characterization of four iPSC lines: two iPSC lines from a mother–child pair (BIHi269-B and BIHi267-B) carrying the *MT-ATP6* mutation m.9176 T > G, and two iPSCs from a mother–child pair (BIHi266-A and BIHi276-A) carrying the *MT-ATP6* mutation m.8993 T > G.

(OCT3/4, TRA1-60, SSEA4) was confirmed by FACS at passage #19 (BIHi266-A) (Fig. 1D), #15 (BIHi276-A), #17 (BIHi267-B, BIHi269-B), #18 (BIHi267-B), and Supplementary files. After formation of embryoid bodies (EBs) at passage #16 (BIHi269-B), #17 (BIHi267-B, BIHi276-A), and #24 (BIHi266-A), iPSCs showed the capacity to differentiate into all three germ layers, which was confirmed by IF staining for the marker proteins of mesoderm (α-smooth muscle actin, SMA and fibronectin, FN), endoderm (α-fetoprotein, AFP and SOX17), and ectoderm (PAX6 and TUJ1) (Fig. 1B, scale bars 300 µm). PCR-restriction fragment length polymorphism (PCR-RFLP) analyses were performed to confirm mtDNA mutations and measure their heteroplasmy levels. We found similar degrees of heteroplasmy in patient fibroblasts and iPSCs (Fig. 1C). In addition, STR analysis confirmed the derivation of the iPSCs from the related source skin fibroblasts. Karyotype analysis performed at passage #16 (BIHi266-A, BIHi267-B, BIHi269-B) or #18 (BIHi276-A) did not show any anomalies (Fig. 1E). Absence of mycoplasma contamination was confirmed for all lines (Supplementary files).

4. Materials and methods

4.1. Testing the iPSC clones for genotype, residual vectors DNA, pluripotency, and viral infection

iPSCs were obtained using Sendai virus based reprogramming (CytoTune-iPS 2.0). Detailed methods, testing for the absence of the reprogramming vectors, FACS staining and analysis for pluripotency markers, and short tandem repeat analysis were done as described

(Hennig et al., 2019). Methods of SNP analysis for karyotyping, and mycoplasma screening were published (Cernoch et al., 2021). Screening of donor cells for HIV1/2 and Hepatitis B/C was done by commercial diagnostic laboratories on a routine basis. Information on antibodies and on primers used is listed in Table 2.

4.2. Differentiation into three germ layers

IPSCs were detached as clusters using Dispase 1:50 in DMEM/F12 (Gibco, 31330–038) and carefully transferred into one well of a non-tissue culture 6-well plate (Falcon, 351146). Cells were cultivated under hypoxic conditions in iPSbrew (Miltenyi Biotec, 130–104-368) at 37 °C in 5 % CO_2 for seven days and daily medium change. During this time, the iPSCs started to form EBs. Forming EBs were then equally distributed over Geltrex-coated (Gibco, A1413302) coverslips placed in 24-well plates. Here, the cells were cultured under normoxic conditions at 37 °C and 5 % CO_2 in EB medium containing DMEM (Gibco, 41966–029) with 20 % FBS (Gibco, 10500–064), 1 % MEM non-essential amino acids solution (Invitrogen, 11140035), 0.1 mM β-Mercaptoethanol (Gibco, 21985–023) with daily medium change (double-feed over the weekend). Formation of EBs was checked daily with an inverted light microscope (Leica DMi1). After seven days, spontaneously differentiated EBs were stained for mesodermal (SMA and Fibronectin) and endodermal (AFP and SOX17) marker proteins (Table 2). Another seven days later, spontaneously differentiated EBs became suitable for staining of ectodermal (TUJ1 and PAX6) marker proteins (Table 2).

4.3. Immunostaining

iPSCs were grown on four sterile coverslips of 14 mm diameter, placed in a well of a 6-well plate and coated with Geltrex (Gibco, A1413302). After formation of suitable colonies, cells were washed once with 1x PBS (Gibco, 14190–094) and fixed with 4 % paraformaldehyde for 15 min at RT. Cells were washed three times with 1x PBS and stored at 4 °C until staining. Fixated iPSCs were incubated in blocking solution consisting of 10 % normal goat (Abcam, ab7481) or donkey serum (Abcam, ab7475) and 1 % Triton X-100 (Sigma, T-9284) in 1x PBS at RT for 60–90 min. The first antibody (Table 2) was applied in blocking solution and incubated at 4 °C O/N. Cells were washed thrice for 5 min with 1x PBS. The secondary antibody (Table 2) was also applied in blocking solution and incubated for 60 min at RT in the dark on a shaking board. After 50 min, DAPI (Invitrogen, D1306, 1:1,000) was added to the antibody mixture and incubated under the same conditions for the remaining 10 min. Cells were washed thrice for 5 min with 1x PBS. Coverslips were dipped into ddH_2O, mounted with 5 µL Mowiol (Roth, 0713) and dried in the dark at RT overnight and were then moved to 4 °C for long-term storage. Fluorescence imaging was performed with Leica DMi8 and Leica application suite X (RRID:SCR_013673).

4.4. Mutation analysis and heteroplasmy measurement

iPSCs and patient fibroblasts were washed with 1x PBS once and scraped with a cell scraper (Sarstedt, 83.3951). The cell suspension was centrifuged at 8000×*g* for 5 min and the supernatant removed. The cell pellet was frozen in liquid nitrogen and stored at −80 °C. Genomic DNA was isolated using the Nucleo-Spin Tissue kit (Macherey-Nagel, 740952.250). DNA concentration was measured with NanoDrop2000c and diluted to 50 ng/µL. The degree of heteroplasmy for the *MT-ATP6* mutations in iPSCs was quantified by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis. For quantification of the m.8993 T > G mutation, a FAM-labeled PCR-fragment was generated with oligonucleotides (Table 2), which was cleaved by *Hpa*II (NEB, R0171) into 25 + 155 bp fragments only in the presence of the mutation. For quantification of the m.9176 T > G mutation, a FAM-labeled PCR-fragment was

Table 2
Reagents details *RRID Requirement for antibodies*: use http://antibodyregistry.org/ to retrieve RRID for antibodies and include ID in table as shown in examples.

Antibodies used for immunocytochemistry/flow-cytometry					
	Antibody	Dilution	Company	Cat #	RRID
Pluripotency markers (IF staining)	mouse anti-OCT3/4 (C10)	1:150	Santa Cruz	sc-5279	RRID:AB_628051
	mouse anti-TRA1-60	1:200	Millipore	MAB4360	RRID:AB_2119183
	goat anti-SOX2	1:20	R&D Systems	AF2018	RRID:AB_355110
Pluripotency markers (FACS)	anti-SSEA4-VioBlue	1:10	Miltenyi Biotec	130–098-366	RRID:AB_2653526
	anti-OCT3/4-APC	1:20	Miltenyi Biotec	130–123-318	RRID:AB_2653086
	anti-TRA1-60-Vio488	1:700	Miltenyi Biotec	130–106-872	RRID:AB_2654228
	anti-NANOG-PE	1:100	Cell Signaling	14955S	N/A
Differentiation markers	mouse anti-TUJ1	1:600	Sigma	T8578	RRID:AB_1841228
	rabbit anti-PAX6	1:50	Invitrogen	42.6600	RRID:AB_2533534
	mouse anti-SMA	1:200	Cell Signaling	48938S	RRID:AB_2799347
	rabbit anti-fibronectin	1 µg/µl	Abcam	ab299	RRID:AB_303474
	rabbit anti-AFP	10 µg/µl	Prosci	16–742	RRID:AB_2904160
	mouse anti-SOX17	1:50	OriGene	CF500044	RRID:AB_2904161
Secondary antibodies	goat anti-mouse 568 IgG2a	1:200	Invitrogen	A21134	RRID:AB_2535773
	goat anti-rabbit 488 IgG	1:200	Invitrogen	A11034	RRID:AB_2576217
Primers					
	Target	Size of band	Forward/Reverse primer (5′-3′)		
Sendai virus vectors	SeV		F: 5-GGA TCA CTA GGT GAT ATC GAG C-3		
			R: 5-ACC AGA CAA GAG TTT AAG AGA TAT GTA TC-3		
	SeV-Klf4		F: 5-TTC CTG CAT GCC AGA GGA GCC C-3		
			R: 5-AAT GTA TGC AAG GTG CTC-3		
	SeV-cMyc		F: 5-TAA CTG ACT AGC AGG CTT GTC G-3		
			R: 5-TCC ACA TAC AGT CCT GGA TGA TGA TG-3		
	SeV-KOS		F: 5-ATG CAC CGC TAC GAG TGA GCG C-3		
			R: 5-ACC TTG ACA ATC CTG ATG TGG-3		
House-keeping genes	human 18S RNA	500 bp	F: 5-GTA ACC CGT TGA ACC CCA TT-3		
			R: 5-CCA TCC AAT CGG TAG TAG CG-3		
mtDNA mutation analysis	m.8993 T > G	180 bp	F: 5-AGC CTA CTC ATT CAA CCA ATA GCC C-3		
			R: FAM-GGC GAC AGC GAT TTC TAG GA-3		
	m.9176 T > G	179 bp	F: FAM-CTG CAG GCC ACC TAC TCA TG-3		
			R: 5-GTC GTG CAG GTA GAG GCT TTC T-3		

generated with oligonucleotides (Table 2), which was cleaved by *Xba*I (NEB, R0145) into 24 + 155 bp fragments only in the presence of the mutation. The percentage of cleaved *versus* uncleaved fragments was determined by capillary electrophoresis and laser detection of the FAM-labelled RFLP-fragment using the 3500 Series Genetic Analyzer (Applied Biosystems, RRID:SCR_021901) and normalized to a standard curve.

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.

Acknowledgments

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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.2023.103030.

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