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Generation of iPSCs from identical twin, one affected by LHON and one unaffected, both carrying a combination of two mitochondrial variants: m.14484 T>C and m.10680G>A

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A B S T R A C T

Leber hereditary optic neuropathy (LHON) is one of the most common mitochondrial illness, causing retinal ganglion cell degeneration and central vision loss. It stems from point mutations in mitochondrial DNA (mtDNA), with key mutations being m.3460G > A, m.11778G > A, and m.14484 T > C. Fibroblasts from identical twins, sharing m.14484 T > C and m.10680G > A variants each with 70 % heteroplasmy, were used to generate iPSC lines. Remarkably, one twin, a LHON patient, displayed symptoms, while the other, a carrier, remained asymptomatic. These iPSCs offer a valuable tool for studying factors influencing disease penetrance and unravelling the role of m.10680G > A, which is still debated.

1. Resource table

Unique stem cell lines identifier	*FINCBi005-A* *FINCBi006-A*
Alternative names of stem cell lines	*Mt1072 #103; #103 (FINCBi005-A)* *Mt1108 #121; #121 (FINCBi006-A)*
Institution	*Fondazione IRCCS Istituto Neurologico C. Besta*
Contact information of distributor	*Valeria Tiranti,*
Type of cell lines	*iPSC*
Origin	*Human*
Cell Source	*Skin fibroblasts*
Clonality	*Clonal*
Method of reprogramming	*Sendai virus (The reprogramming vectors include the four Yamanaka factors, Oct3/4, Sox2, Klf4, and L-Myc)*
Multiline rationale	*Same disease but one patient affected and one carrier unaffected, twins with same nuclear and mitochondrial DNA*
Gene modification	*YES*
Type of modification	*Hereditary*
Associated disease	*LHON*
Gene/locus	*Heteroplasmic m.14484 T > C (M64V) on locus MT-ND6 and heteroplasmic m.10680G > A (A71T) on locus MT-ND4L*
Method of modification	*N/A*
Name of transgene or resistance	*N/A*
Inducible/constitutive system	*N/A*
Date archived/stock date	*December 2022*
Cell line repository/bank	*https://hpscreg.eu/cell-line/FINCBi005-A* *https://hpscreg.eu/cell-line/FINCBi006-A*
Ethical approval	*Fondazione IRCCS Istituto Neurologico Carlo Besta, Ethical Committee approval: n.60, 6th March 2019*

2. Resource utility

The two iPSC lines are generated from two individuals with the combination of the heteroplasmic mtDNA variants m.14484 T > C and m.10680G > A. They are identical twins, one of whom is affected by

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Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
FINCBi005-A	*#103*	*Male*	*39*	*Caucasian*	*Homoplasmic m.10680G > A* *Homoplasmic m.14484 T > C*	*LHON*
FINCBi006-A	*#121*	*Male*	*39*	*Caucasian*	*Heteroplasmic m.10680G > A (4 %)* *Heteroplasmic m.14484 T > C (8 %)*	*LHON, carrier*

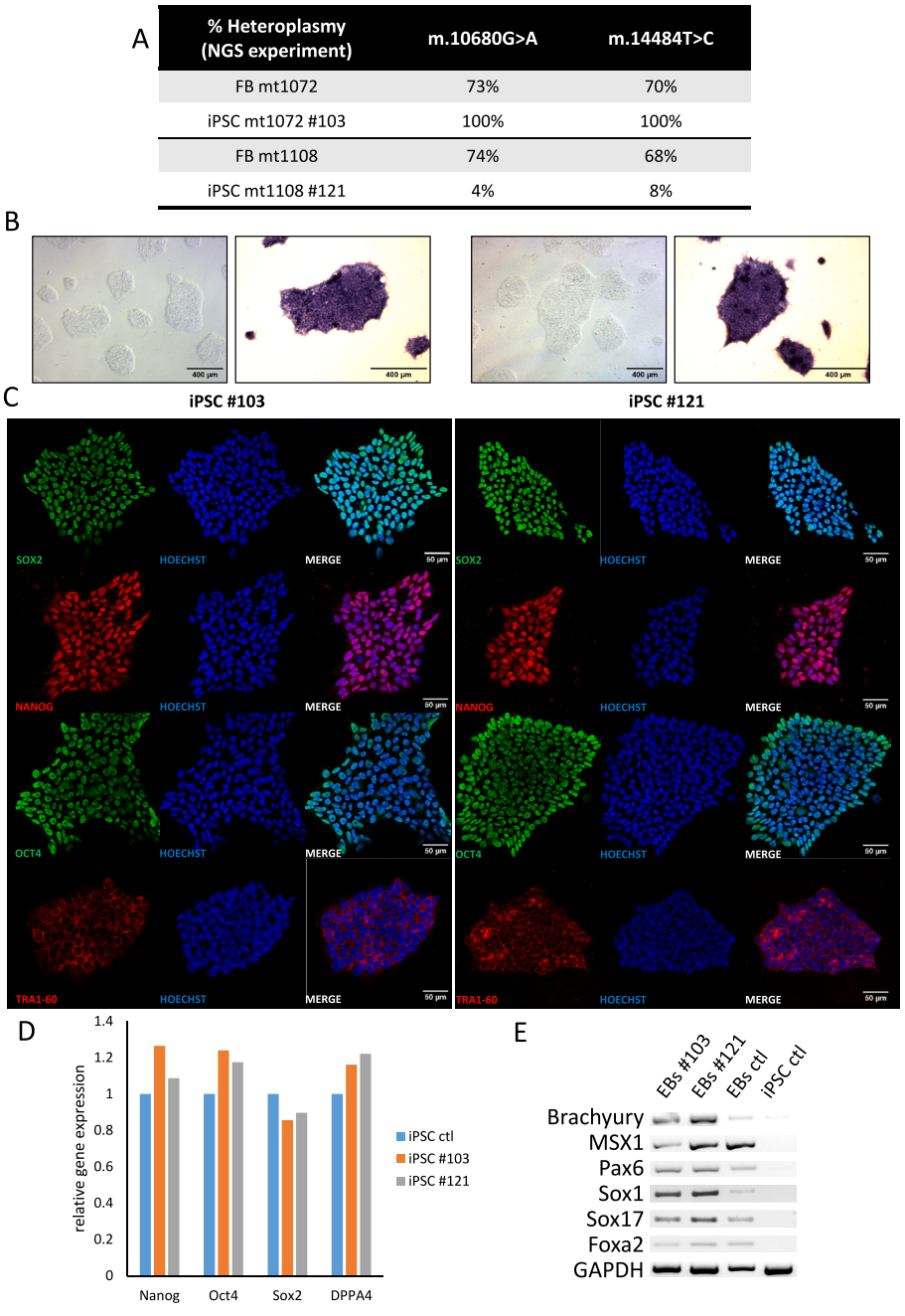

Fig. 1.

LHON and the other is an unaffected carrier of both variants (see Table 1).

3. Resource Details

Among mitochondrial diseases, Leber hereditary optic neuropathy (LHON) is one of the most prevalent (Schaefer et al., 2008). As a result of retinal ganglion cell degeneration, one or both eyes may lose their central vision. The disease is caused by point mutations in the mitochondrial DNA (mtDNA), specifically one of the three most common variants: m.3460G > A, m.11778G > A, and m.14484 T > C. These variants are usually homoplasmic but some of the patients have a

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	*Normal*	Fig. 1B
Phenotype	Alkaline Phosphatase Staining	*Staining resulted positive*	Fig. 1B
	Immunocytochemistry	*Staining of pluripotency markers SOX2; NANOG;* OCT4; TRA1-60	Fig. 1C
	RT-PCR	*Expression of pluripotency markers OCT4, SOX2, DPPA4, NANOG*	Fig. 1D
		Loss of Sendai virus expression (SeV)	Supplementary Fig. 1C
Genotype Identity	CGH array	*arr(1–22)x2, (X,Y)x1, normal male*	Supplementary Fig. 1A
	Sequencing	*Whole mtDNA genome sequencing by NGS*	https://doi.org/10.5281/zenodo.10406130
	STR	*7 loci analyzed, all matching*	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	*Heteroplasmic mtDNA point mutations*	Supplementary Fig. 1B
	Southern Blot OR WGS	*N/A*	N/A
Microbiology and virology	Mycoplasma	*Mycoplasma testing by MycoAlert was Negative*	Supplementary Fig. 1D
Differentiation potential	Embryoid body formation	*Expression of three germ-layers markers MSX1 and BRACHYURY (mesoderm), PAX6 and SOX1 (ectoderm), FOXA2 and SOX17 (endoderm)*	Fig. 1E
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

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	Mouse anti-NANOG	1:200	Sigma-Aldrich Cat# N3038, RRID:AB_1079449
	Rabbit anti-SOX2	1:200	Abcam Cat# ab97959, RRID: AB_2341193
	Mouse anti-TRA1-60	1:200	Abcam Cat# ab16288, RRID: AB_778563
	Rabbit anti-OCT4	1:200	Abcam Cat# ab19857, RRID: AB_445175
Secondary antibodies	Goat anti-Mouse IgG Alexa Fluor 546	1:1000	Thermo Fisher Scientific Cat# A-11030, RRID:AB_2534089
	Goat anti-Rabbit IgG Alexa Fluor 488	1:1000	Thermo Fisher Scientific Cat# A-11034, RRID:AB_2576217
	Hoechst 33,342	1:1000	Thermo Fisher Scientific Cat #H-1399
Primers			
	Target	**Forward/Reverse primer (5′-3′)**	
Virus loss expression	Sendai Virus (SeV)	GGATCACTAGGTGATATCGAGC / ACCAGACAAGAGTTTAAGAGATATGTATC	
Pluripotency Markers	qNANOG	CCTGTGATTTGTGGGCCTG / GACAGTCTCCGTGTGAGGCAT	
	qOCT4	GTGGAGGAAGCTGACAACAA / ATTCTCCAGGTTGCCTCTCA	
	qSOX2	GTATCAGGAGTTGTCAAGGCAGAG / TCCTAGTCTTAAAGAGGCAGCAAAC	
	qDPPA4	TGGTGTCAGGTGGTGTGTGG / CCAGGCTTGACCAGCATGAA	
House-Keeping Gene	GAPDH	GTGTGAACCATGAGAAGTATGACAAC / CTTCACCACCTTCTTGATGTCATC	
	qACTIN	CCAACCGCGAGAAGATGA / CCAGAGGCGTACAGGGAT	
Differentiation Markers (mesoderm)	MSX1	CGAGAGGACCCCGTGGATGCAGAG/ GGCGGCCATCTTCAGCTTCTCCAG	
	BRACHYURY	GCTGAAGGTGAACGTGTCTG / ACCGCTATGAACTGGGTCTC	
Differentiation Markers (ectoderm)	PAX6	GTAGCGACTCCAGAAGTTGT / AGTTTATCATACATGCCGTCTGC	
	SOX1	AGATGCACAACTCGGAGATC / GCCAGCGAGTACTTGTCCTT	
Differentiation Markers (endoderm)	FOXA2	GGAGCGGTGAAGATGGAA / TACGTGTTCATGCCGTTCAT	
	SOX17	CTCTGCCTCCTCCACGAA / CAGAATCCAGACCTGCACAA	
Long range PCR for NGS	Whole mtDNA	CCGCACAAGAGTGCTACTCTCCTC / GATATTGATTTCACGGAGGATGGTG	
Short Tandem Repeats Markers	D1S80	GAAACTGGCCTCCAAACACTGCCCGCCG / GTCTTGTTGGAGATGCACGTGCCCCTTGC	
	ApoB (Chr 2)	ATGGAAACGGAGAAATTATG / CCTTCTCACTTGGCAAATAC	
	D10S1214 (Chr 10)	ATTGCCCCAAAACTTTTTTG / TTGAAGACCAGTCTGGGAAG	
	D11S533 (Chr 11) (4F7)	GCCTAGTCCCTGGGTGTGGTC / GGGGGTCTGGGAACATGTCCCC	
	D17S1290 (Chr 17)	GCAACAGAGCAAGACTGTC / GGAAACAGTTAAATGGCCAA	
	D19S894 (Chr 19)	TTACTTGGCCCCAGGAAGC / GTTAAGCCATAAACATGGAATGACC	
	D21S2055 (Chr 21)	AACAGAACCAATAGGCTATCTATC / TACAGTAAATCACTTGGTAGGAGA	

heteroplasmic condition. The carriers are those individuals who did not experience any symptom while having the LHON mutation.

We generated iPSC lines from two twins carrying a combination of the mitochondrial point mutations m.14484 T > C (M64V) on locus MT-ND6 associated with LHON and the variant m.10680G > A (A71T) on locus MT-ND4L, whose role is controversial (Yang et al., 2009; Zhang et al., 2012). One of the twins is an affected LHON patient and the other is an unaffected LHON carrier. Both patient fibroblasts are heteroplasmic for both mutations with a percentage of about 70 % each (Fig. 1A) and share haplogroup X2d1.

We collected fibroblasts and reprogrammed them into iPSCs using the Sendai virus. Among the obtained clones, we characterised one clone derived from the LHON patient (mt1072, clone #103) and one clone derived from of the unaffected carrier (mt1108, clone #121) (Table 2). Both clones have normal morphology and are positive for Phosphatase Alkaline staining (Fig. 1B). To confirm the stemness of the iPSCs, we performed immunofluorescence experiments and tested the presence of the pluripotency factors SOX2, NANOG, OCT4, and TRA1-60 (Fig. 1C) and gene expression of NANOG, OCT4, SOX2 and DPPA4 by qRT-PCR (Fig. 1D).

The genomic integrity of the two clones was verified using array Comparative Genomic Hybridization (CGH) and both resulted in a normal male karyotype (Supplementary Fig. 1A). We also sequenced the whole mitochondrial DNA (mtDNA) by NGS to ensure that no additional new mutations appeared. In addition, the entire mtDNA sequence was

used to verify the match between the clones and their corresponding starting fibroblasts (https://doi.org/10.5281/zenodo.10406130). This correspondence was also confirmed by testing seven different short tandem repeat (STR).

We monitored the percentage of heteroplasmy for the two variants in our iPSCs by NGS. The clone derived from the LHON patient resulted homoplasmic for both the variants. The LHON carrier clone resulted heteroplasmic with a 4 % of the m.10680G > A and an 8 % of the m.14484 T > C (Fig. 1A and Supplementary Fig. 1B). Additional clones, either from LHON patients and unaffected carrier with different percentages of the two variants were identified but not fully characterised.

After a few passages, we checked for the absence of the Sendai virus in our iPSCs (Supplementary Fig. 1C).

The differentiation potential of the iPSCs was examined by generating embryoid bodies. RT-PCR was used to measure the expression of the three germ-layer markers, which are: FOXA2 and SOX17 for endoderm, PAX6 and SOX1 for ectoderm, and MSX1 and BRACHYURY for mesoderm (Fig. 1E).

Mycoplasma tests were performed on each clone, and the results were negative (Supplementary Fig. 1D).

All the experiments were done with cells at passage 7–10.

4. Materials and Methods

4.1. iPSCs generation and culture

We generated iPS cells from skin fibroblasts using the CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Life Technologies).

iPSCs were cultured on 1:100 Cultrex™ Stem cell qualified Reduced Growth factor basement membrane matrix (Trevigen) in Essential 8™ Flex medium (Gibco) at 37 °C and 5 % CO_2, and passaged as clumps with 0,5mM EDTA at a ratio of 1:3–1:6 with 10 μM Y27632 (DBA) twice a week.

Little clusters of colonies were grown in suspension in Essential 8 medium plus 1X N2 Supplement (Life Technologies), 5 ng/ml Noggin Recombinant Protein (Tebu Bio), 10 μM SB431542 (Sigma), and 10 μM Y27632 (only day 1) to obtain embryoid bodies (EBs). Medium was changed every other day for up to 4–8 days. To create rosette-like forms, we plated EBs on a 1:100 Cultrex™ Stem cell for 4–8 days.

4.2. Alkaline phosphatase staining

We fixed IPSC with 2 % PFA for 10 min at room temperature. Cells were then incubated with the Sigma*fast*™ BCIP®/NBT substrate (Sigma) for 20 min and visualized with a phase-contrast microscope.

4.3. Immunofluorescence

We fixed iPSCs with 4 % Paraformaldehyde (for SOX2, NANOG and OCT4) for 20 min at room temperature or cold EtOH (for TRA1-60) for 15 min at −20 °C. Cells were permeabilized in 0.5 % Triton for 10 min and then, after an hour at room temperature in a blocking solution (PBS + 10 % NGS + 1 % BSA), they were incubated with primary antibodies (Table 3) in PBS + 3 % NGS + 1 % BSA overnight at 4 °C. Subsequently, the colonies were incubated with secondary antibodies (Table 3) and observed using a Leica TCS SP8 confocal microscope.

4.4. RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Quiagen) and *retro*-transcribed with the GoTaq®2-Step RT-qPCR System (Promega). Real-Time PCR was performed with iTaq Universal SYBR Green Supermix (Bio-Rad) on a CFX96 Touch Real-Time PCR detection System (Bio-Rad). Data were normalized to the housekeeping gene Actin and relative expression of the markers was compared with a published iPSC line (FINCBi004-A) (Zanuttigh et al., 2023). Primers used are in Table 3.

4.5. CGH

We performed array Comparative Genomic Hybridization (CGH) on DNA extracted from cultured iPS cells (about $1x10^6$ cells) using DNA extraction kit (Gentra kit, Qiagen, Hilden, Ge) as already described (Peron et al., 2020).

4.6. Sequencing

DNA was extracted using Phenol-Chloroform protocol and UltraRun LongRange PCR Kit (Qiagen) was employed to obtain whole mtDNA sequence. We diluted PCR products to a final concentration of 0.2 ng/μl and processed according to Nextera XT DNA Library Prep protocol (Illumina). Indexed DNA libraries were pooled together with equal molar ratios and sequenced on MiSeq platform with a v3 Illumina Flowcell (600 cycles) and a paired-end read chemistry.

4.7. Mycoplasma

We used MycoAlert Mycoplasma Detection Kit (Lonza).

Credit authorship contribution statement

Camille Peron: Writing – original draft, Methodology, Formal analysis. **Andrea Cavaliere:** Investigation. **Chiara Fasano:** Investigation. **Angelo Iannielli:** Investigation. **Manuela Spagnolo:** Investigation. **Andrea Legati:** Investigation. **Maria Nicol Colombo:** Investigation. **Ambra Rizzo:** Investigation. **Francesca L. Sciacca:** Investigation. **Valerio Carelli:** Funding acquisition. **Vania Broccoli:** Funding acquisition. **Costanza Lamperti:** Resources. **Valeria Tiranti:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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.

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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.2024.103406.

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