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Lab Resource: Single Cell Line

Generation and characterization of CSSi016-A (9938) human pluripotent stem cell line carrying two biallelic variants in MTMR5/SBF1 gene resulting in a case of severe CMT4B3

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ABSTRACT

Charcot-Marie-Tooth type 4B3 (CMT4B3) is a rare subtype of hereditary neuropathy associated with variants in the MTMR5/SBF1 gene. Herein, we report the generation and characterization of a hiPSC line from a 12-year-old Italian girl with early onset severe polyneuropathy with motor and axonal involvement, harboring biallelic variants in the MTMR5/SBF1 gene. Fibroblasts were reprogrammed using non-integrating episomal plasmids, and iPSCs successfully passed the stemness and pluripotency tests. Patient-specific hiPSCs were produced to obtain a disease model for the study of this rare condition.

1. Resource Table:

Unique stem cell line identifier	CSSi016-A (9938)
Alternative name(s) of stem cell line	*PORA cl C*
Institution	IRCCS Casa Sollievo della Sofferenza
Contact information of distributor	Jessica ROSATI; j.rosati@css-mendel.it
Type of cell line	*hiPSC*
Origin	*Human*
Additional origin info requiredfor human ESC or iPSC	*Age: 12Sex: femaleEthnicity if known: Caucasian/Italian*
Cell Source	*Dermal Fibroblasts*
Clonality	*Clonal*
Method of reprogramming	*Non integrating episomal vectors*
Genetic Modification	*NO*
Type of Genetic Modification	*NO*
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	*qRT-PCR*
Associated disease	*Charcot-Marie-Tooth disease (CMT) type 4B3 (CMT4B3)*
Gene/locus	MTMR5/SBF1 gene; chromosome 22, position 50,898,793c.3194G > A; c.3191G > A p.G1064E;MTMR5/SBF1 gene; chromosome 22, position 50,900,742c.2288G > A; c.2291G > A p.R763H
Date archived/stock date	*December 2021*
Cell line repository/bank	https://hpscreg.eu/cell-line/CSSi016-A
Ethical approval	*IRCCS Fondazione Stella Maris, Comitato Etico Pediatrico, regione Toscana, CEP:102/2020*

1.1. Resource utility

CMT4B3, among the rarest autosomal recessive forms of the disease, is caused by variants in myotubularin-related proteins, namely MTMR5/SBF1. We generated iPSCs which will be used for studying in vitro the molecular mechanisms underlying the disease. Table 1.

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2. Resource details

The commonest hereditary motor and sensory neuropathies (HMSN) is the Charcot-Marie-Tooth disease (CMT) with a prevalence of 17–40 per 100,000 individuals (Nagappa et al., 2021). The clinical features include progressive distal muscle atrophy and weakness, both in the lower limbs and later also the upper extremities, decreased or absent tendon reflexes, foot deformities, distal sensory loss and steppage gait. CMT is associated with more than 30 loci and about 20 causative genes are known thus far (Higuchi and Takashima, 2022). A subtype of Charcot-Marie-Tooth is the type 4B3 (CMT4B3) that is associated with variants in the myotubularin-related 5 (MTMR5) also called SET binding factor 1 (SBF1) gene (Nakhro et al., 2013). Dermal fibroblasts were obtained from a skin biopsy from a 12-year-old Italian girl with progressive and severe infantile axonal motor neuropathy, harboring biallelic variants in the MTMR5/SBF1 gene, p.R763H (c.2291G > A) and p.G1064E (c.3194G > A) resulting in a case of severe CMT4B3 (Berti et al., 2021). We reprogrammed fibroblasts using non-integrative episomal vectors containing the reprogramming factors OCT4, SOX2, L-MYC, KLF4, LIN28, and shp53. iPSC colonies (Fig. 1A) were manually picked and expanded in culture. The colonies with uniform, flat and stem cell-like morphology were selected for characterization (Fig. 1B). To verify the genomic stability, we analyzed the iPSC karyotype, which was confirmed after XVI passage as a normal karyotype (46, XX) (Fig. 1C). Genomic DNA Sequencing analysis confirmed the presence of the disease-related mutation (p.R763H; c.2291G > A and p.G1064E; c.3194G > A) in iPSCs (Fig. 1D). Immunofluorescence analysis revealed the expression of the surface marker TRA-1–60 and the transcription factor OCT4 in CMT4B3 colonies (Fig. 1E): TRA-1–60 (red signal) is present in the cells surface and OCT4 (green signal) protein is evident in the nuclei of the iPSCs cells. After XV passage of amplification in vitro, we confirmed the suppression of exogenous reprogramming factors expression through qPCR analysis using primers against episomal sequence flanking the exogenous genes, (Fig. 1F), the positive control was constituted by nucleofected fibroblasts. The expression of endogenous pluripotency markers KLF4, LIN28, OCT4, L-MYC and SOX2 were detected by qRT-PCR after fifteen passages, using fibroblast cells as a negative control of the pluripotency marker expression and an already published line of hiPSCs (CSS012-A(7672))(D'Anzi et al., 2021) as reference control (Fig. 1G). To assess the pluripotency capacity of the CMT4B3 iPSCs we performed two different tests: on one hand, in vitro test through embryoid bodies formation assay and, on the other hand, in vivo test through the teratoma assay. The iPSCs spontaneously aggregate into embryoid bodies (EBs) (Fig. 1H) and after fourteen days in the culture, they expressed the markers of the three germ layers (Fig. 1I). iPSCs were inoculated in the immunodeficient mice and successfully generated a teratoma composed by all three germ layers as demonstrated by immuno-histochemical analysis (Fig. 1J). Finally, short tandem repeats analysis (STR) showed that the DNA profile of parental fibroblasts was the same of the derived iPSCs (data available by the authors). All cells were periodically tested negative for Mycoplasma contamination (Supplementary File 1).

3. Materials and methods

3.1. Fibroblast culture and reprogramming method

Dermal fibroblasts from the CMT4B3 patient were grown in Dulbecco's Modified Eagle Medium High Glucose with 20 % FBS, 2 mM L-Glutamine, 100 U/ml Penicillin-Streptomicin and 1 % Non-Essential Amino Acids (SigmaAldrich) at 37 °C and 5 % CO_2. About 1×10^5 fibroblasts, passage VI, were nucleofected with 3ug of 1:1:1 episomal mix of pCXLE-hUL (Addgene#27080), pCXLE-hSK (Addgene#27078) and pCXLE-hOCT4-shp53 (Addgene#27077) using 4D-Nucleofector™ (Lonza), FF113 program and P2 buffer. One week later, fibroblasts were plated on a dish

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	Normal	Fig. 1 panel B
Phenotype	Immunocytochemistry	Staining of pluripotency markers: Oct4 and Tra1-60	Fig. 1 panel E
	Quantitative analysis RT-qPCR	Expression of pluripotency markers: OCT4, LIN28, L-MYC, SOX2	Fig. 1 panel G
Genotype	Karyotype (G-banding) and resolution	46XX, Resolution 450–500	Fig. 1 panel C
Identity	STR analysis	All the 17 sites tested matched	submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	Sbf1-19F 5′-TATGGGGATGTGCAGACTCA-3′ Sbf1-19R 5′-ACTCTCCGTGCAGACCTTGT-3′; Sbf1-25F 5′-TCCCTCAGGTATGGATCTGG-3′ Sbf1-25R 5′-CTCCCTGGCCAATGTCAG-3′.	Fig. 1 panel D
Microbiology and virology	Mycoplasma	Mycoplasma tested by N-Garde Mycoplasma PCR kit (EuroClone) is Negative	Fig. 1 supplementary
Differentiation potential	Embryoid body formation and Teratoma formation	Embryoid bodies morphology, Genes expressed in embryoid bodies: SOX1, NESTIN, PAX6, EOMES, T, GATA4, FOXA2, SOX17 Proof of teratoma three germ layers formation.	Fig. 1 panel H, Fig. 1 panel I Fig. 1 panel J
List of recommended germ layer markers	**Expression of these markers has to be demonstrated at mRNA (RT PCR) or protein (IF) levels, at least 2 markers need to be shown per germ layer**	Ectoderm: SOX1, NESTIN, PAX6, FABP, SLC1A3; Endoderm: SOX17, GATA4, FOXA2, Mesoderm: EOMES, T	Fig. 1 panel I qRT-PCR with reference gene(s)
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	
	HLA tissue typing	N/A	

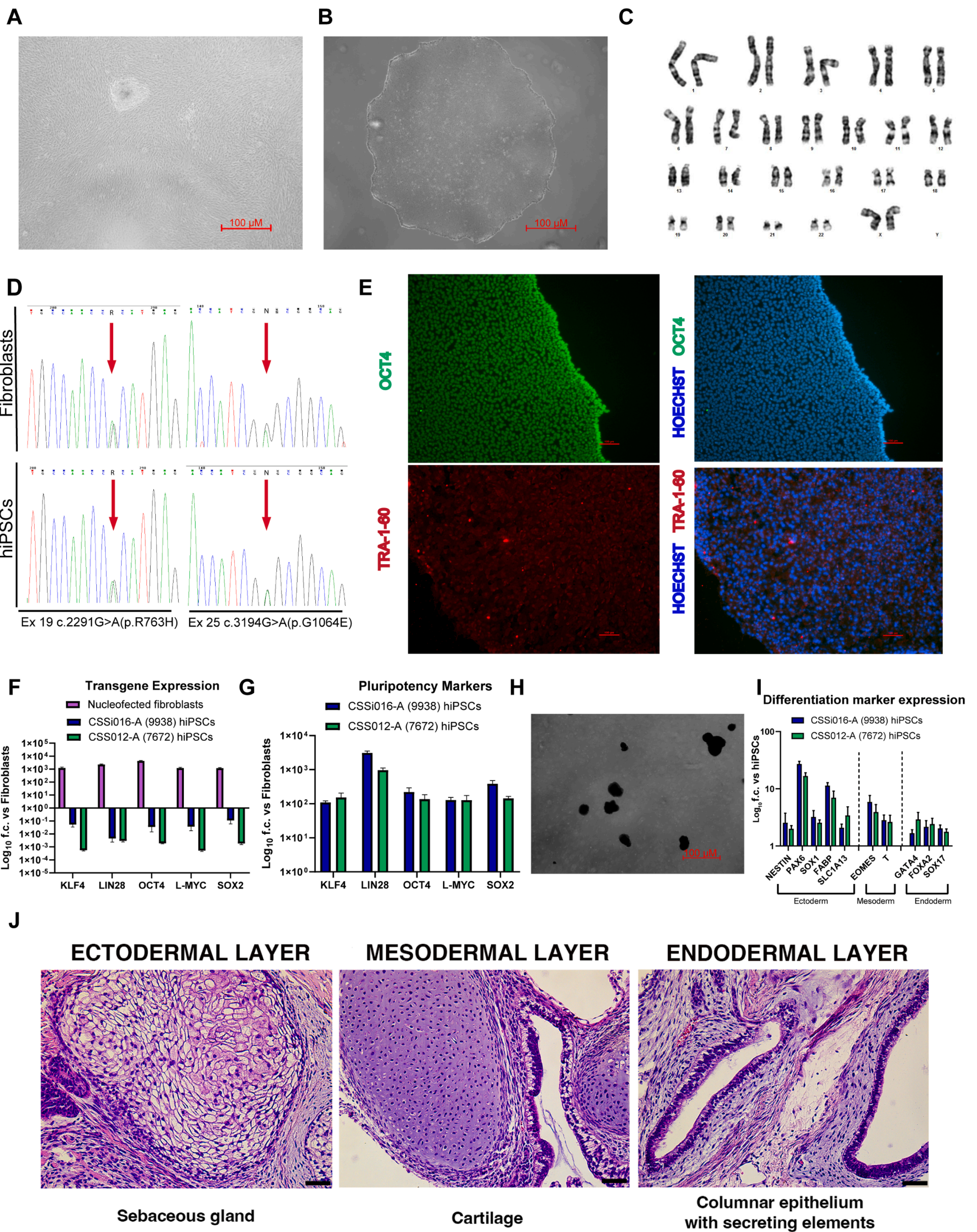

Fig. 1.

pretreated with Matrigel (Corning) and cultured in NutristemXF medium (Biological industries). Colonies with uniform and flat stem-cell-like morphology were picked and expanded one time at week at 37 °C and 5 % CO2. iPSCs were tested periodically for the absence of mycoplasma contamination using an N-Garde Mycoplasma PCR kit.

3.2. qPCR analyses

Total RNAs from parental, nucleofected fibroblasts and iPSCs, XII passage, were isolated using TRIzol reagent (Life Technologies). After validation of RNA integrity through RNA 6000 Nano LabChips (Agilent Technologies), processed on the Agilent 2100 Bioanalyzer, cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). SYBR Green primers for pluripotency and TaqMan primers for differentiation (Table 2) were used to perform qRT-PCR, analyzed through the 2–ΔΔCT method. Each reaction was run in triplicate using β-ACTIN as a reference gene.

3.3. Karyotype analysis

iPSCs were cultured in Nutristem XF medium for 2–3 days and then treated with a 0.1 µg/ml COLCEMID solution (Thermo Fisher Scientific) for 60 min at 37 °C. Metaphases were obtained and karyotype analysis was carried out on GTG-banding. Thirty metaphases were counted, and three karyotypes were analyzed.

3.4. Immunofluorescence staining

iPSCs were fixed using 4 % paraformaldehyde for 20 min at RT. Clones were blocked in PBS containing 20 % Normal Goat Serum for TRA-1–60 staining and the same solution with 0.1 % Triton X-100 for OCT4 staining, for 30 min at RT. Primary antibodies, listed in Table 2, diluted in blocking buffer were incubated O/N at 4 °C. Alexa-Fluor-conjugated secondary antibodies were added for 1 h at RT. Cellular nuclei were counterstained with Hoechst. Images were taken using a Nikon C2 fluorescence microscope.

3.5. Pluripotency in vitro and in vivo assay

IPSCs, at XII passage, were picked up and transferred in 25 cm flasks in floating conditions. Nutristem-XF medium was gradually switched with DMEM F-12, 20 % Knock-out serum replacement (Gibco), 0.1 mM β-mercaptoethanol, 1 × NEAA, 50 U/ml Penicillin-Streptomycin, 2 mM L-glutamine. After fourteen days, EBs were collected. To evaluate the in vivo teratoma formation, iPSCs derived from six-well plates, combined with 100ul Matrigel, were injected into the flank of nude mice. Once visible, teratomas were collected for histological analysis with hematosilin/eosin staining.

3.6. STR analysis

*Dneasy blood and tissue kit (QIAGEN) were used for DNA extraction. PCR amplification of 17 distinct STRs was carried out using the QST*Rplusv2 kit (Elucigene Diagnostics), then PCR products were separated on an ABI Prism 3130 DNA sequencer and analyzed by GeneMapper version 4.0 (AppliedBiosystems). STRs: AMEL, D3S1358, TH01, D21S11, D18S51, D10S1248, D1S1656, D2S1338, D16S539, D22S1045, vWA, D8S1179, FGA, D2S441, D12S391, D19S433, SE33.*

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Table 2
Reagents details.

	Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat #**	**RRID**
Pluripotency Markers	*Rabbit anti-OCT4;*	*1:100*	*Life technologies (A13998);*	*RRID: AB 2534182;*
	Mouse anti-TRA-1–60	*1:100*	*Life technologies (411000)*	*RRID: AB_2533494*
Secondary antibodies	*Anti-Rabbit AlexaFluor 488;*	*1:1000*	*Invitrogen (A11034);*	*RRID: AB_2576217;*
	Anti-Mouse AlexaFluor 555	*1:1000*	*Invitrogen (A21422).*	*RRID: AB_2535844*
	Primers			
SYBR green Primers used for qPCR	**Target**	**Size of band**	**Forward/Reverse primer (5′-3′)**	
Episomal Plasmids (qPCR)	*eOCT4*	*70–150 bp*	*Fwd: CAT TCA AAC TGA GGT AAG GG Rev: TAG CGT AAA AGG AGC AAC ATA G*	
	eLIN28	*70–150 bp*	*Fwd: AGC CAT ATG GTA GCC TCA TGT CCG C Rev: TAG CGT AAA AGG AGC AAC ATA G*	
	eL-MYC	*70–150 bp*	*Fwd: GGC TGA GAA GAG GAT GGC TAC Rev: TTT GTT TGA CAG GAG CGA CAA T*	
	eSOX2	*70–150 bp*	*Fwd: TTC ACA TGT CCC AGC ACT ACC AGA Rev: TTT GTT TGA CAG GAG CGA CAA T*	
	eKLF4	*70–150 bp*	*Fwd: CCA CCT CGC CTT ACA CAT GAA GA Rev: TAG CGT AAA AGG AGC AAC ATA G*	
Pluripotency Markers (qPCR)	*OCT4*	*70–150 bp*	*Fwd: CCC CAG GGC CCC ATT TTG GTA CC Rev: ACC TCAGTT TGA ATG CAT GGG AGA GC*	
	LIN28	*70–150 bp*	*Fwd: CCC CAG GGC CCC ATT TTG GTA CC Rev: ACC TCA GTT TGA ATG CAT GGG AGA GC*	
	L-MYC	*70–150 bp*	*Fwd: GCG AAC CCA AGA CCC AGG CCT GCT CC Rev: CAG GGG GTC TGC TCG CAC CGT GAT G*	
	SOX2	*70–150 bp*	*Fwd: TTC ACA TGT CCC AGC ACT ACC AGA Rev: TCA CAT GTG TGA GAG GGG CAG TGT GC*	
House-Keeping Genes (qPCR)	*β-ACTIN*	*70–150 bp*	*Fwd: GGC ATC CTC ACC CTG AAG TA*	

(continued on next page)

Table 2 (*continued*)

	Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat #**	**RRID**
			Rev: GGG GTG TTG AAG GTC TCA AA	
TaqMan primers used for qPCR		***Target***		***Probe***
Differentation markers	*SOX1*		*Hs01057642_s1*	
	NESTIN		*Hs04187831_g1*	
	PAX6		*Hs00240871_m1*	
	T		*Hs00610080_m1*	
	EOMES		*Hs00172872_m1*	
	GATA4		*Hs00171403_m1*	
	FOXA2		*Hs00232764_m1*	
	SOX17		*Hs00751752_s1*	
	β-ACTIN		*Hs 99999903_m1*	
Targeted mutation analysis/ sequencing	**MTMR5/ SBF1**		*Sbf1-19F 5′-TATGGGGATGTGCAGACTCA-3′* *Sbf1-19R 5′-ACTCTCCGTGCAGACCTTGT-3′;* *Sbf1-25F 5′-TCCCTCAGGTATGGATCTGG-3′* *Sbf1-25R 5′-CTCCCTGGCCAATGTCAG-3′.*	

and FMS).

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

No data was used for the research described in the article.

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This paper is dedicated to the loving memory of AP who recently passed away. We thank the family for participating in this study, the patients' associations MITOCON, UILDM (Unione Italiana Lotta alla Distrofa Muscolare), and CollaGe-Associazione-Genitori-Manzoni-Poli-Molfetta.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2022.102946.

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