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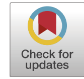
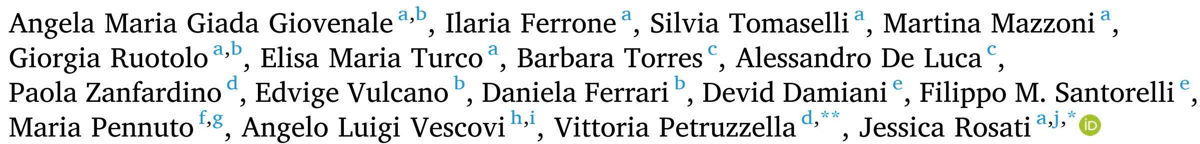
Lab Resource: Single Cell Line



Generation and characterization of a patient-derived iPSC line, CSSi022-A (15666), with a pathogenic MFN2 mutation causing Charcot-Marie-Tooth disease type 2A



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ABSTRACT

Charcot-Marie-Tooth disease type 2A (CMT2A; OMIM 609260) is a rare sensorimotor neuropathy caused by mutations in the MFN2 gene (1p36.22). We successfully reprogrammed fibroblasts from an 8-year-old girl carrying a de novo MFN2 mutation into induced pluripotent stem cells using non-integrative vectors. The line shows normal karyotype, pluripotency, and trilineage differentiation, providing a valuable in vitro model to study disease mechanisms.

Resource Table

Unique stem cell line identifier	CSSi022-A (15666)
Alternative name(s) of stem cell line	CMT2A-C217F cl T
Institution	Fondazione IRCCS Casa Sollievo della Sofferenza
Contact information of distributor	Jessica Rosati; j.rosati@operapadrepio.it
Type of cell line	
Origin	Human
Additional origin info required for human ESC or iPSC	Age: 8 Sex: female Ethnicity: 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 TYPE 2A
Gene/locus	NM_001127660.2c.[650G>T]
Date archived/stock date	March 2021
Cell line repository/bank	https://hpscreg.eu/cell-line/CSSi022-A
Ethical approval	IRCCS Fondazione Stella Maris, Comitato Etico Pediatrico, regione Toscana, CEP:102/2020

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1. Resource utility

CHARCOT-MARIE-TOOTH DISEASE TYPE 2A (CMT2A) is a rare inherited autosomal dominant condition associated with a mutation in the mitofusin 2 (MFN2) gene. We generated a stable, patient-specific hiPSC line from an 8-year-old female to establish a disease-specific cellular model to study and clarify the underlying molecular mechanisms.Table 1.

2. Resource details

Charcot-Marie-Tooth disease (CMT) encompasses a variety of motor and sensory neuropathies, commonly referred to as hereditary motor-sensory neuropathies (HMSN). CMT can be categorized into different types based on the specific genes involved. Monoallelic mutations in the Mitofusin 2 gene (MFN2) are associated with Charcot-Marie-Tooth disease type 2A (CMT2A). This subtype is characterized by progressive distal weakness, sensory loss, decreased or absent tendon reflexes, and foot deformities ("Hereditary Neuropathy," 2023; Okamoto & Takashima, 2023).

We conducted reprogramming of primary dermal fibroblasts obtained from a skin biopsy of an 8-year-old girl who carries the monoallelic variant c.650G>T (p.Cys217Phe) in the MFN2 gene, which causes CMT2A (Zanfardino et al., 2023). The reprogramming utilized non-integrative episomal vectors to express the reprogramming factors OCT4, SOX2, L-MYC, KLF4, and LIN28. We selected clones based on their stem cell-like morphology (Fig. 1A) and verified their normal karyotype (46, XX) after passage VI (PVI) (Fig. 1B). Genomic DNA sequencing confirmed the presence of the same MFN2 mutation (c.650G>T) in both the induced pluripotent stem cells (hiPSCs) and the primary fibroblasts (Fig. 1C).

After PVI, we characterized the hiPSCs for expression of pluripotency markers. Immunostaining demonstrated the presence of the cytoplasmic marker TRA-1–60 (red) and the nuclear marker OCT4 (green) (Fig. 1D). Additionally, through RT-PCR, we confirmed the expression of pluripotency markers, including OCT4, SOX2, L-MYC, KLF4, and LIN28 in the hiPSCs (Fig. 1E), while noting the absence of transgene expression (Fig. 1F). For all RT-PCR analyses, we used the CSSi018-A (14192) hiPSC line as a standard reference, as previously published (Casamassa et al., 2024). To further assess the pluripotency of the generated hiPSC line, we performed two distinct assays. First, the hiPSCs were cultured under floating conditions, resulting in the spontaneous formation of human induced embryoid bodies (hiEBs) (Fig. 1G). After 14 days of culture, we evaluated the hiEBs for the expression of differentiation markers specific to the three embryonic layers (Fig. 1H). Pluripotency was also assessed *in vivo* using a teratoma formation assay, which revealed the presence of all three embryonic layers upon histological analysis (Fig. 1I). Furthermore, short tandem repeats (STR) analysis confirmed that the DNA profiles of the parental fibroblasts and the derived hiPSCs were identical (data available upon request). Finally, we regularly tested the cells and confirmed they were negative for Mycoplasma contamination (Supplementary File 1).

3. Materials and methods

3.1. Fibroblast culture and reprogramming method

Dermal fibroblasts were cultured in Dulbecco's Modified Eagle Medium with high glucose, supplemented with 20 % fetal bovine serum (FBS), 1 % L-glutamine, non-essential amino acids (NEAA), and penicillin–streptomycin (Sigma Aldrich). The cells were maintained at 37 °C in a 5 % CO_2 environment. A total of 3×10^5 fibroblasts at passage VI were nucleofected with 1.5 μg of a 1:1:1 episomal mix containing pCXLE-hUL (Addgene #27080), pCXLE-hSK (Addgene #27078), and pCXLE-hOCT4-shp53 (Addgene #27077) using a 4D-Nucleofector™ (Lonza Program FF113). One week later, the nucleofected fibroblasts were plated on a dish pretreated with Matrigel (Corning) and cultured in NutristemXF medium (Biological Industries). Colonies exhibiting stem cell-like morphology were amplified at 37 °C in a 5 % CO_2 environment. Induced pluripotent stem cells (iPSCs) were periodically tested for mycoplasma contamination using an N-Garde Mycoplasma PCR kit.

3.2. qPCR analyses

Total RNA was isolated using TRIzol reagent (Life Technologies). After validating RNA integrity with Nano LabChips (Agilent Technologies) and processing samples on the Agilent 2100 Bioanalyzer, RNA was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). SYBR Green primers for pluripotency and TaqMan primers for differentiation (as listed in Table 2) were utilized to perform quantitative real-time PCR (qRT-PCR), which was analyzed using the $2^{-\Delta\Delta CT}$ method. Each reaction was conducted in triplicate, with β-ACTIN used as a reference gene.

3.3. Karyotype and STR analysis

For karyotyping, iPSCs at passage IX were cultured in Nutristem medium for 2–3 days. Karyotype analysis was carried out on GTG-banded metaphases (resolution 450–500). 30 metaphases were counted and three karyograms were analyzed. For STR analysis, the DNA was extracted using Dneasy blood and tissue kit (QIAGEN). PCR amplification of 17 distinct STRs was carried out using the QST*Rplusv2 kit

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	Normal	Fig. 1, panel A
Phenotype	Immunocytochemistry	Staining of stemness markers: Oct4 and Tra1-60	Fig. 1, panel D
	Quantitative analysis RT-qPCR	Expression of stemness markers: OCT4, LIN28, L-MYC, SOX2, KLF4	Fig. 1, panel E-F
Genotype	Karyotype (G-banding) and resolution	46 XX,Resolution 450–500	Fig. 1, panel B
Identity	STR analysis	All 17 sites tested matched	submitted to the archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	NM_001127660.2c.[650G>T]	Fig. 1, panel C
Microbiology and virology	Mycoplasma	Mycoplasma tested by the N-Garde Mycoplasma PCR kit (EuroClone) isNegative	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 G Fig. 1, panel H Fig. 1, panel I
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	

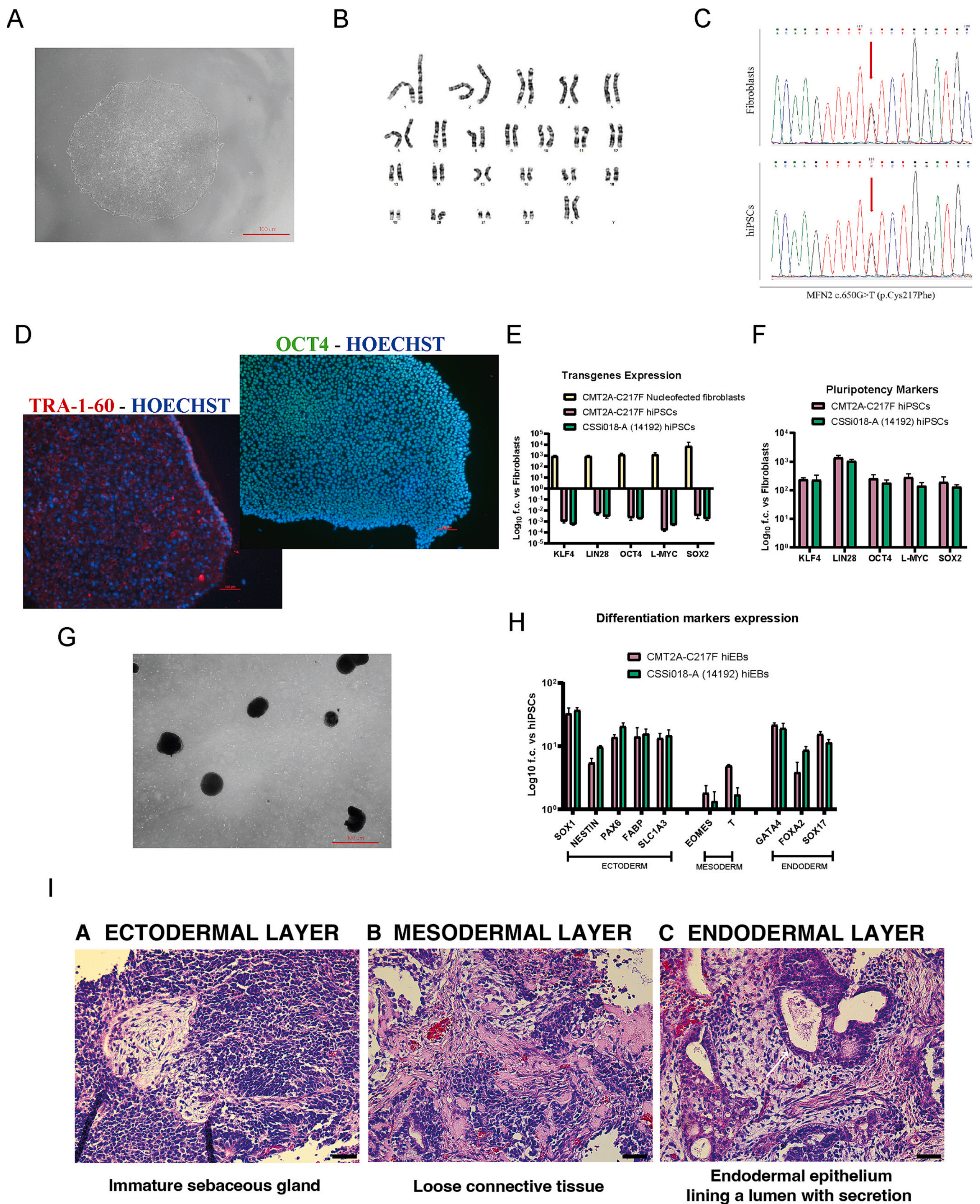

Fig. 1.

Table 2
Reagents details.

	Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat #	RRID
Pluripotency Markers	Rabbit anti-OCT4; Mouse anti-TRA-1–60	1:100 1:100	Life technologies (A13998); Life technologies(411000)	RRID: AB 2534182;RRID: AB_2533494
Secondary antibodies	Anti-Rabbit AlexaFluor 488; Anti-Mouse AlexaFluor555	1:1000 1:1000	Invitrogen (A11034); Invitrogen(A21422)	RRID: AB_2576217; RRID: AB_2535844
	Primers			
SYBR green Primers used for qRT-PCR	**Target**	**Amplicon size**	**Forward/Reverse primer (5′-3′)**	
Episomal Plasmids (qRT-PCR)	eOCT4	83 bp	Fwd: CAT TCA AAC TGA GGT AAG GG Rev: TAG CGT AAA AGG AGC AAC ATA G	
	eLIN28		Fwd: AGC CAT ATG GTA GCC TCA TGT CCG C	
	eL-MYC	80 bp	Rev: TAG CGT AAA AGG AGC AAC ATA G Fwd: GGC TGA GAA GAG GAT GGC TAC	
	eSOX2	66 bp	Rev: TTT GTT TGA CAG GAG CGA CAA T	
	eKLF4	112 bp	Fwd: TTC ACA TGT CCC AGC ACT ACC AGA Rev: TTT GTT TGA CAG GAG CGA CAA T Fwd: CCA CCT CGC CTT ACA CAT GAA GA Rev: TAG CGT AAA AGG AGC AAC ATA G	
Stemness Markers (qRT-PCR)	OCT4	179 bp	Fwd: TTG CTG CAG AAG TGG GTG GA Rev: TGG CTG ATC TGC TGC AGT GT	
	LIN28	169 bp		
			Fwd: TGA GAG GCG GCC AAA AGG AA	
	L-MYC		Rev: CAG CGG ACA TGA GGC TAC CA	
		142 bp		
			Fwd: GCG AAC CCA AGA CCC AGG CCT GCT CC Rev: CAG GGG GTC TGC TCG CAC CGT GAT G	
	SOX2			
		80 bp	Fwd: TTC ACA TGT CCC AGC ACT ACC AGA Rev: ACC TCA GTT TGA ATG CAT GGG AGA GC	
	KLF4			
		166 bp	Fwd: TCT CAA GGC ACA CCT GCG AA Rev: CCT GGA AAA TGC TCG GTC GC	
House-Keeping Genes (qRT-PCR)	β-ACTIN	203 bp	Fwd: GGC ATCCTC ACC CTGAAG TARev: GGG GTGTTG AAG GTCTCA AA	
TaqMan primers used for qRT-PCR	**Target**		**Probe**	
Differentationmarkers	SOX1 NESTIN PAX6 T EOMES GATA4 FOXA2 SOX17β-ACTIN		Hs01057642_s1 Hs04187831_g1 Hs00240871_m1 Hs00610080_m1 Hs00172872_m1 Hs00171403_m1 Hs00232764_m1 Hs00751752_s1Hs 99999903_m1	
Targeted mutation analysis/sequencing	MFN2	326 bp	Fwd: TCC CTG GCT TTC TCC TCC ATG Rev: AAG GGT AAG ACC TCG GTG GAG	

(Elucigene Diagnostics), then PCR products were separated on an ABI Prism 3130 DNA sequencer and analyzed by GeneMapper version 4.0 (Applied Biosystems) (Table STR).

3.4. Immunofluorescence staining

iPSCs were fixed using 4 % paraformaldehyde for 20 min at RT. Clones were blocked in PBS with 20 % Normal Goat Serum for TRA-1–60 staining and with the addition of 0.1 % Triton X-100 for OCT4 staining, for 1 h at RT. Primary antibodies (Table 2), diluted in blocking buffer were incubated O/N at 4 °C. Alexa-Fluor-conjugated secondary antibodies were added for 1,5 h at RT. Cellular nuclei were stained with Hoechst. Images were taken using a Nikon C2 fluorescence microscope.

3.5. Pluripotency assay

IPSCs, after XIV passage, were transferred in floating conditions in 25 cm flasks. Nutristem-XF medium was gradually switched to 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 14 days, hiEBs were collected and characterized. To evaluate the *in vivo* teratoma formation, iPSCs combined with 100ul Matrigel were injected into the flank of immunodeficient mice. After teratoma formation, the histological analysis was performed via hematosilin/eosin staining.

3.6. Sequencing

Polymerase chain reaction (PCR) was performed to amplify a specific region including the mutation, with the specific primers (See Table 2), using Wonder Taq Polymerase (EuroClone) and analyzed by Sanger Sequencing at Microsynth Seqlab GmbH (Göttingen, Germany).

CRediT authorship contribution statement

Angela Maria Giada Giovenale: Data curation, Writing – original draft. **Ilaria Ferrone:** Data curation. **Silvia Tomaselli:** Data curation. **Martina Mazzoni:** Data curation. **Giorgia Ruotolo:** Data curation. **Elisa Maria Turco:** Data curation. **Barbara Torres:** Data curation. **Alessandro De Luca:** Data curation. **Paola Zanfardino:** Data curation.

Edvige Vulcano: Data curation. **Daniela Ferrari:** Data curation. **Devid Damiani:** Data curation. **Filippo M. Santorelli:** Data curation. **Maria Pennuto:** Data curation. **Angelo Luigi Vescovi:** Supervision. **Vittoria Petruzzella:** Writing – review & editing. **Jessica Rosati:** Conceptualization, Data curation, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jessica Rosati reports financial support was provided by Ministry of Health.

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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.2025.103817.

Data availability

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

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