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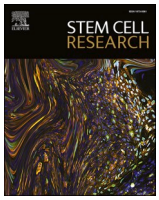

Production of an induced pluripotent stem cell line CSSi018-A (14192) from a patient with hypomyelinating leukodystrophy 7 (HLD7) carrying biallelic variants of POLR3A (c.1802 T > A; c.4072G > A)

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A B S T R A C T

Hypomyelinating leukodystrophies (HLD) are a group of heterogeneous genetic disorders characterized by a deficit in myelin deposition during brain development. Specifically, 4H-Leukodystrophy is a recessive disease due to biallelic mutations in the POLR3A gene, which encodes one of the subunits forming the catalytic core of RNA polymerase III (PolIII). The disease also presents non-neurological signs such as hypodontia and hypogonadotropic hypogonadism. Here, we report the generation of a human induced pluripotent stem cell (hiPSC) line from fibroblasts of the first identified carrier of the biallelic POLR3A variants c.1802 T > A and c.4072G > A.

Resource Table:

Unique stem cell line identifier	CSSi018-A (14192)
Alternative name(s) of stem cell line	IPS cl P
Institution	IRCCS Casa Sollievo della Sofferenza
Contact information of distributor	Jessica ROSATI; j.rosati@css-mendel.it
Type of cell line	iPSC
Origin	human
Additional origin info required for human ESC or iPSC	Age at skin biopsy: 10 years Sex: F Ethnicity if known: Caucasian/Italian
Cell Source	Dermal Fibroblasts
Clonality	Clonal
Method of reprogramming	Non integrating episomal vectors
Genetic Modification	Yes
Type of Genetic Modification	Congenital
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	qRT-PCR
Associated disease	Hypomyelinating Leukodystrophy (HLD7)
Gene/locus	RNA polymerase III subunit A (POLR3A): c.1802 T > A; c.4072G > A
Date archived/stock date	April 2022
Cell line repository/bank	https://hpscreg.eu/cell-line/CSSi018-A
Ethical approval	*Casa Sollievo della Sofferenza Ethical Committee, approval number: 136/CE*

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1. Resource utility

The hiPSC line described here was derived from a 4H Leucodystrophy patient carrying the biallelic POLR3A variants c.1802 T > A and c.4072G > A, both of which have not yet been reported in the literature. The differentiation of this line will be useful as experimental model to study the pathogenesis of the disease.

2. Resource details

Hypomyelinating leukodystrophies (HLDs) is a group of heterogeneous genetic disorders characterized by a deficiency in myelin deposition during brain development. These diseases are rare, affecting one out of every 250,000 to 500,000 people (Sawaguchi et al., 2021). This recessive disorder is associated with mutations of the RNA polymerase 3 subunit A (*POLR3A*) gene, encoding for one of the catalytic subunits of Pol III, enzyme responsible for the synthesis of small non-coding RNAs including 5S rRNA, snRNAs, tRNAs and miRNAs (Sawaguchi et al., 2021). *POLR3A* mutations lead to a dysfunction of Pol III, which affects the expression of tRNAs that are more abundant in the CNS, and thus disrupts protein synthesis (Ruan et al., 2024).

Here we describe the production of an induced pluripotent stem cell line, generated from skin fibroblasts obtained from a 10-year-old patient carrying the biallelic POLR3A variants c.1802 T > A and c.4072G > A, not previously reported in the literature (Table 1). She was the second son of non-consanguineous healthy Italian parents. Her older brother was diagnosed with POLR3A leukodystrophy. Neonatal period was normal with the correct acquisition of cognitive and motor milestones. At age 5 years the patient manifested cognitive and motor regression. Neurodegeneration progressed over time with development of spasticity of the limbs, loss of head control at age 10 and onset of dysphagia. Skin fibroblasts from the proband were reprogrammed into hiPSCs, using three non-integrative episomal vectors containing the reprogramming factors *OCT 3/4, SOX2, L-MYC, KLF4, LIN28* and p53-shRNA (Addgene). HLD7 hiPSCs colonies at passage VII displayed a normal diploid 46, XX chromosome arrangement (Fig. 1A) and typical morphology of human stem cells, flattened with well-defined contours (Fig. 1B). Immunostaining for endogenous markers TRA-1-60 and OCT-4 demonstrated the pluripotency of these cells at passage XII (Fig. 1C), also confirmed through qRT-PCR using fibroblasts as negative control and CSSi013-A (9360) hiPSCs as positive control (Fig. 1D; D'Anzi et al., 2022). We confirmed the absence of episomal plasmid DNA in the established hiPSCs line, after nine passages, using nucleofected fibroblasts as positive control and CSSi013-A (9360) hiPSCs as negative control, through qRT-PCR (Fig. 1E). The presence of the biallelic *POLR3A* variants c.1802 T > A and c.4072G > A detected in parental fibroblasts was confirmed by Sanger sequencing (Fig. 1F). The capability to differentiate in the three embryonic germ layers, at passage XIV, was demonstrated *in vitro* through the embryoid bodies (EBs) assay (Fig. 1G), and *in vivo* through the teratoma formation (Fig. 1 I). In addition, Fig. 1H shows the endogenous mRNA expression of the three germ layers markers in the EBs compared to the previously published line. PCR-based detection tests confirmed the absence of Mycoplasma contamination at this stage (Supplementary Fig. 1). Finally, Short Tandem Repeat (STR) profiling confirmed that this hiPSC line had the same genetic identity with respect to the donor's fibroblasts.

3. Materials and methods

3.1. Fibroblasts culture and reprogramming

Fibroblasts obtained from the processing of the patient's skin biopsy were cultured in high-glucose DMEM supplemented with 20 % FBS, 100 U/ml Penicillin-Streptomycin, 2 mM L-Glutamine, 1 × non-essential amino acids (all Sigma Aldrich reagents), at 37 °C, 5 % CO2. Using Nucleofector4D (Lonza Amaxa), 3 × 105 fibroblasts at passage 6 were electroporated with 3 μg of 1:1:1 mixture of the episomal plasmids pCXLE-hUL (Addgene #27080), pCXLE-hSK (Addgene #27078) and pCXLE-hOCT4-shp53 (Addgene #27077). After seven days, nucleofected fibroblasts were counted and plated on Matrigel-coated dishes (BD Biosciences) with Nutristem XF medium (Biological Industries). The hiPSC colonies were collected and expanded under non-feeding conditions. The absence of mycoplasma contamination was verified using the N-Garde Mycoplasma kit (EuroClone).

3.2. Embryoid body and teratoma formation assays

Mechanically detached hiPSCs (step XIV) were placed in a 25 mM flask under floating conditions. Nutristem-XF medium was gradually changed in DMEM/F-12, 20 % Knock-out replacement serum (Gibco), 0.1 mM β-mercaptoethanol, 0.1 mM NEAA, 1 % penicillin–streptomycin and 1 % glutamine. Fourteen days later, EBs were pelleted and RNAs were extracted. For teratoma formation, dyspase-treated hiPSCs derived from 6-well plates were resuspended in 100 μl Matrigel and injected into

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	Normal	Fig. 1B
Phenotype	Qualitative analysis: immunocytochemistry	Staining of pluripotency markers: OCT4; TRA-1-60	Fig. 1C
	Quantitative analysis: RT-qPCR	Expression of pluripotency markers: *KLF4*, *LIN28*, *L-MYC*, *OCT4*, *SOX2*	Fig. 1D
Genotype	Karyotype (G-banding) and resolution	46 XX, Resolution 450–500	Fig. 1A
Identity	STR analysis	All the 17 sites tested matched	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	*POLR3A*: c.1802 T > A and 4072G > A in heterozygosis	Fig. 1F
Microbiology and virology	Mycoplasma	Mycoplasma tested by N-Garde Mycoplasma PCR kit (Euroclone). Negative	Supplementary Fig. 1
Differentiation potential	Embryoid body formation Teratoma formation	Genes expressed in embryoid bodies and three germ layers in teratoma	Fig. 1G and Fig. 1I
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* Mesoderm: *EOMES*, *T* Endoderm: *GATA4*, *FOXA2*, SOX17	Fig. 1H
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	

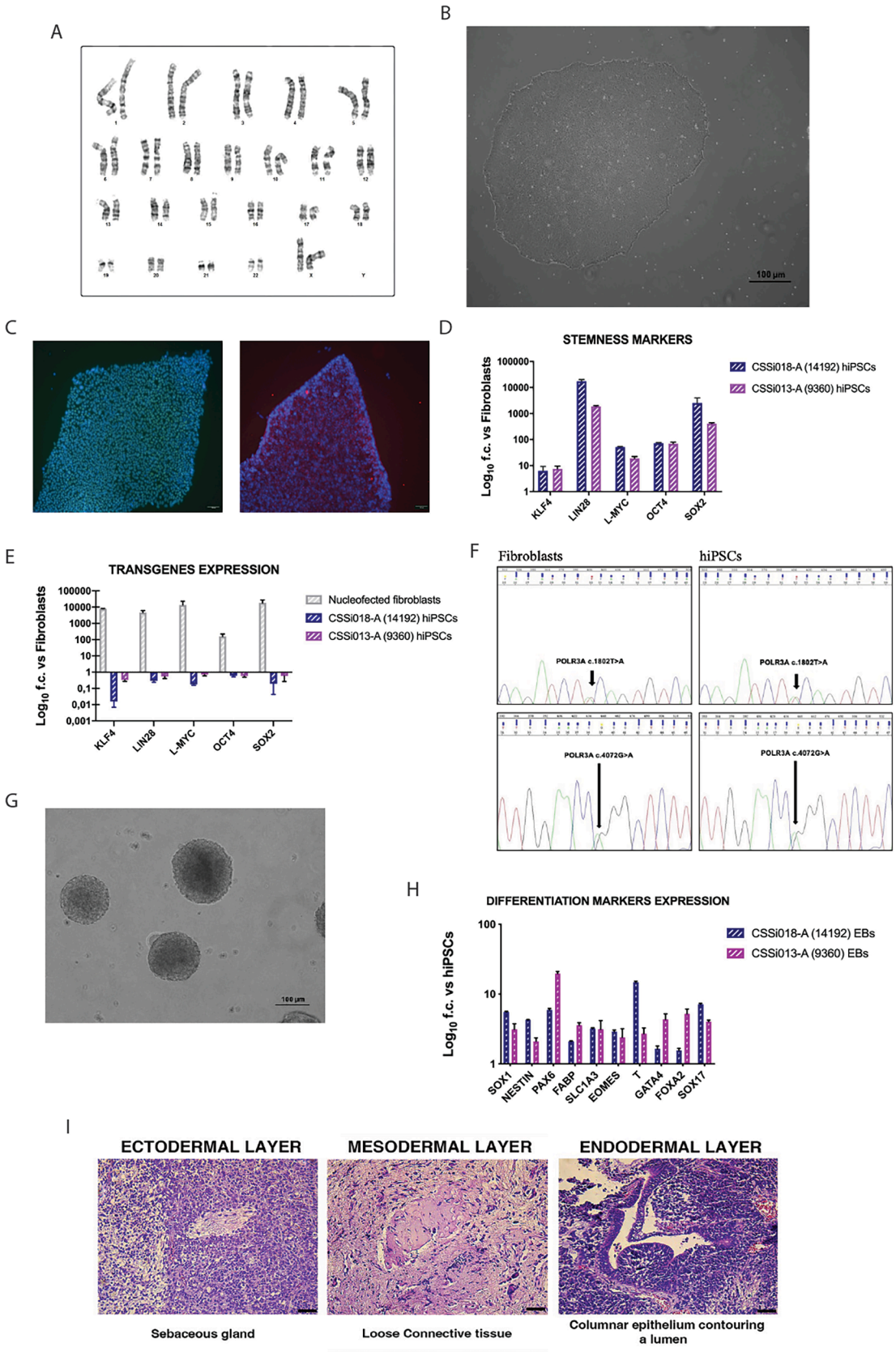
A
B
100 µm
C
D
STEMNESS MARKERS
CSSi018-A (14192) hiPSCs
CSSi013-A (9360) hiPSCs
Log10 f.c. vs Fibroblasts
KLF4
LIN28
L-MYC
OCT4
SOX2
E
TRANSGENES EXPRESSION
Nucleofected fibroblasts
CSSi018-A (14192) hiPSCs
CSSi013-A (9360) hiPSCs
F
Fibroblasts
hiPSCs
POLR3A c.1802T>A
POLR3A c.4072G>A
G
100 µm
H
DIFFERENTIATION MARKERS EXPRESSION
CSSi018-A (14192) EBs
CSSi013-A (9360) EBs
Log10 f.c. vs hiPSCs
SOX1
NESTIN
PAX6
FABP
SLC1A3
EOMES
T
GATA4
FOXA2
SOX17
I
ECTODERMAL LAYER
MESODERMAL LAYER
ENDODERMAL LAYER
Sebaceous gland
Loose Connective tissue
Columnar epithelium contouring a lumen

Fig. 1.

NOD/SCID mice according to ethical guidelines. After 9 weeks, the teratomas were removed for histological analysis with haematoxylin/eosin.

3.3. Real-time PCR analysis

Total RNAs were isolated using TRIzol reagent (Life Technologies) according to manufacturer's instructions and cDNA synthesized using the High-capacity cDNA RT (Life Technologies). For pluripotency markers expression we used Power SYBR Green PCR Master Mix (Applied Biosystem), while differentiation markers expression was analyzed through TaqMan Universal PCR Master Mix (Applied Biosystem) (primers listed in Table 2). The expression ratio of target genes was calculated by using the $2^{-\Delta\Delta Ct}$ method, considering *β-ACTIN* as reference gene. qPCR analysis was performed in a technical triplicate, in three independent biological experiments. The already published hiPSC line CSSi013-A (9360) was used as control.

3.4. STR analysis

DNA from Fibroblasts and hiPSCs were extracted with the Dneasy kit for blood and tissue (QIAGEN). PCR amplification of 17 distinct STRs (AMEL, D3S1358, TH01, D21S11, D18S51, D10S1248, D1S1656, D2S1338, D16S539, D22S1045, vWA, D8S1179, FGA, D2S441, D12S391, D19S433, SE33) was carried out using the QST*Rplus v2 kit (Elucigene Diagnostics). PCR products were separated on an ABI Prism 3130 DNA sequencer and analyzed by GeneMapper version 4.0 (Applied Biosystems).

3.5. Sequencing

Genomic DNA was extracted from iPSCs and fibroblasts (VI passage) using ReliaPrep™ Blood gDNA Miniprep System. POLR3A exon 14 was amplified by PCR using Forward: 5-GACTCTCAGGCTTCCTCCC-3, Reverse: 5-GGCACAGAGATCTTCCCCTT-3′ primers; POLR3A exon 31 was amplified by PCR using Forward: 5-TTCTGGTTGGTTTCAGGGGT-3, Reverse: 5-GGGGATGTGGAATTCATTTGTG-3′ primers. The amplicons (product size: 156 bp and 153 bp, respectively) were sequenced by BigDye terminator v.3.1 Cycle Sequencing kit on ABI 3130XL Genetic Analyzer.

3.6. Karyotype analysis

hiPSCs (passage VII) were cultured in Nutristem XF medium for 2–3 days. Cells were treated with a 0.1 μg/mL COLCEMID solution (Thermo Fisher Scientific) for 60 min at 37 °C, 30 mM KCl in 10 %FBS at 37 °C for 6 min and coldfresh-made 3:1 ethanol:acetic acid solution. Karyotyping of metaphase chromosomes was performed using G banding. twenty metaphases were counted.

3.7. Immunofluorescence staining

Cells (passage XII) were fixed using 4 % paraformaldehyde for 20 min at RT and blocked in PBS with 20 % Normal Goat Serum for 30 min (0.1 % Triton X-100 only for OCT4 staining). Primary antibodies diluted in 5 % BSA were incubated overnight at 4 °C. After washing, Alexa Fluor 594- and/or Alexa Fluor 488-conjugated secondary antibodies were added 1 h at RT. Cellular nuclei were counterstained with Hoechst 33,342 (Thermo Fisher Scientific). Microphotographs were taken using a Nikon C2 fluorescence microscope and NIS Elements 1.49 software.

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) *e.g. Biotium inc Cat# 20,010*	RRID: AB_2534182 RRID: AB_2533494
Secondary antibodies	anti-Rabbit AlexaFluor 488; anti-Mouse AlexaFluor 594	1:1000	Invitrogen (A11034); Invitrogen (A21422)	RRID: AB_2576217 RRID: AB_2535844
	Primers			
	Target	**Size of band**	**Forward/Reverse primer (5′-3′)**	
Episomal Plasmids (qPCR)	eOCT4 eKLF4 eLIN28 eL-MYC eSOX2	83 bp 112 bp 205 bp 80 bp 66 bp	Fwd: CAT TCA AAC TGA GGT AAG GG Rev: TAG CGT AAA AGG AGC AAC ATA G Fwd: CCA CCT CGC CTT ACA CAT GAA GA Rev: TAG CGT AAA AGG AGC AAC ATA G Fwd: AGC CAT ATG GTA GCC TCA TGT CCG C Rev: TAG CGT AAA AGG AGC AAC ATA G Fwd: GGC TGA GAA GAG GAT GGC TAC Rev: TTT GTT TGA CAG GAG CGA CAA T Fwd: TTC ACA TGT CCC AGC ACT ACC AGA Rev: TTT GTT TGA CAG GAG CGA CAA T	
Pluripotency Markers (qPCR)	Oct4 LIN28 L-MYC SOX2 KLF4	179 bp 169 bp 142 bp 80 bp 166 bp	Fwd: TTG CTG CAG AAG TGG GTG GA Rev: TGG CTG ATC TGC TGC AGT GT Fwd: TGA GAG GCG GCC AAA AGG AA Rev: CAG CGG ACA TGA GGC TAC CA Fwd: GCG AAC CCA AGA CCC AGG CCT GCT CC Rev: CAG GGG GTC TGC TCG CAC CGT GAT G Fwd: TTC ACA TGT CCC AGC ACT ACC AGA Rev: ACC TCA GTT TGA ATG CAT GGG AGA GC Fwd: TCT CAA GGC ACA CCT GCG AA Rev: CCT GGA AAA TGC TCG GTC GC	
House-Keeping Genes (qPCR)	β-ACTIN	203 bp	Fwd: GGC ATCCTC ACC CTGAAG TA Rev: GGG GTGTTG AAG GTCTCA AA	
Differentation markers	SOX1 NESTIN PAX6 T EOMES GATA4 FOXA2 SOX17 β-ACTIN		Hs01057642_s1 Hs04187831_g1 Hs00240871_m1 Hs00610080_m1 Hs00172872_m1 Hs00171403_m1 Hs00232764_m1 Hs00751752_s1 Hs99999903_m1	

CRediT authorship contribution statement

Alessia Casamassa: Formal analysis, Writing – original draft. **Giovannina Rotundo:** Data curation. **Chiara Ceresoni:** Validation. **Elisa Maria Turco:** Data curation. **Isabella Torrente:** . **Ornella Candido:** . **Francesco Nicita:** Data curation, Resources. **Davide Tonduti:** Resources. **Enrico Bertini:** . **Massimo Marano:** Resources. **Daniela Ferrari:** Data curation. **Cristina Cereda:** Data curation. **Maria Pennuto:** Writing – review & editing. **Angelo Luigi Vescovi:** Writing – review & editing. **Stephana Carelli:** Data curation. **Jessica Rosati:** Conceptualization, Writing – original draft, 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, article publishing charges, and equipment, drugs, or supplies were provided by Ministry of Health. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2024.103468.

References

D'Anzi, A., Perciballi, E., Ruotolo, G., et al., 2022. Production of CSSi013-A (9360) iPSC line from an asymptomatic subject carrying an heterozygous mutation in TDP-43 protein. Stem Cell Res. 63, 102835 https://doi.org/10.1016/j.scr.2022.102835.

Ruan, D.D., Ruan, X.L., Wang, R.L., et al., 2024. Clinical phenotype and genetic function analysis of a family with hypomyelinating leukodystrophy-7 caused by POLR3A mutation. Sci. Rep. 14 (1), 7638. https://doi.org/10.1038/s41598-024-58452-6. Published 2024 Apr 1.

Sawaguchi, S., Tago, K., Oizumi, H., et al., 2021. Hypomyelinating Leukodystrophy 7 (HLD7)-Associated Mutation of POLR3A Is Related to Defective Oligodendroglial Cell Differentiation, Which Is Ameliorated by Ibuprofen. Neurol. Int. 14 (1), 11–33. https://doi.org/10.3390/neurolint14010002.