



Lab Resource: Single Cell Line

Induced pluripotent stem cell production (CSSi019-A)(14432) from an asymptomatic subject carrying a expansion of C9orf72 gene

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ABSTRACT

One of the genetic mutations most associated with the onset of amyotrophic lateral sclerosis, both in sporadic and familial cases, is the expansion of the C9orf72 gene. The presence of more than 30 repeats (GGGGCC) correlates with uncertain ALS symptomatology. Here we collected a dermal biopsy from a subject carrying 36 hexanucleotide repeats and reprogrammed it into an induced pluripotent stem cell line. Despite the number of repeat elements, the subject had no symptoms at the age of the biopsy (76 years), thus resulting in a healthy carrier of the mutation.

1. Resource table

Unique stem cell line identifier	CSSi019-A (14432) https://hpscereg.eu/cell-line/CSSi019-A
Alternative name(s) of stem cell line	A1150 Asymptomatic cl 7
Institution	Fondazione 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: 76 Sex: Male Ethnicity if known: Caucasian
Cell Source	Dermal fibroblasts
Clonality	Clonal
Method of reprogramming	Non-integrating episomal vectors
Genetic Modification	Yes
Type of Genetic Modification	Congenital

(continued on next column)

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Unique stem cell line identifier	CSSi019-A (14432) https://hpscereg.eu/cell-line/CSSi019-A
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	qRT-PCR
Associated disease	Amyotrophic lateral sclerosis
Gene/locus	C9ORF72: c.-45 + 258_45 + 263delGGGGCC[36]
Date archived/stock date	29/11/2022
Cell line repository/bank	https://hpscereg.eu/cell-line/CSSi019-A
Ethical approval	Comitato Etico Università Cattolica del Sacro Cuore A.1320/CE/2012

2. Resource utility

ALS is a multifactorial disease in which genetics and environment

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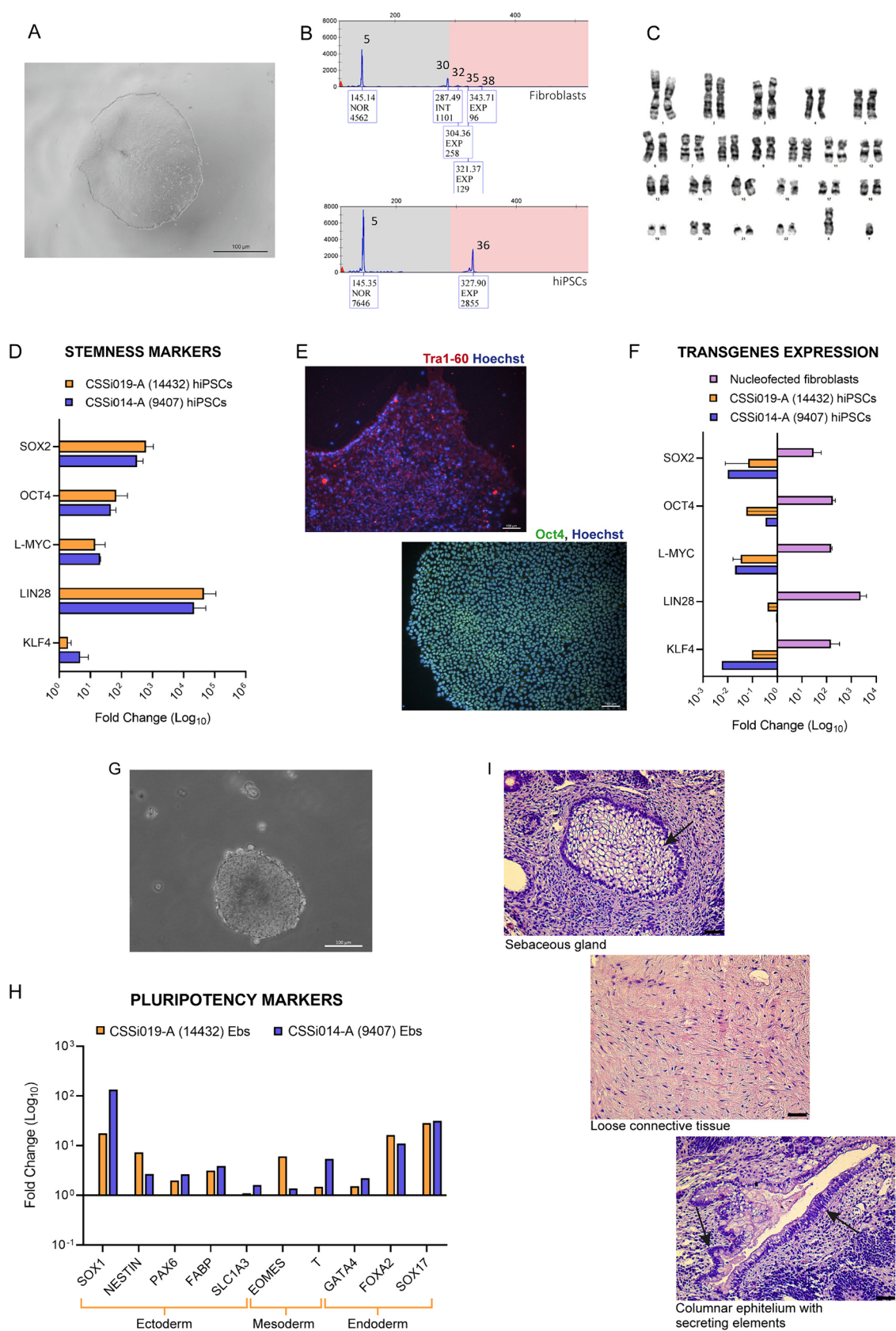


Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology Phenotype	Photography	<i>Normal</i>	Fig. 1 panel A
	Qualitative analysis <i>Immunocytochemistry</i>	<i>Staining of pluripotency markers: Oct4, Tra1-60</i>	Fig. 1 panel E
	Quantitative analysis <i>RT-qPCR</i>	<i>Expression of stemness markers: KLF4, LIN28, L-MYC, OCT4, SOX2</i>	Fig. 1 panel D
Genotype	Karyotype (G-banding) and resolution	<i>46XY, Resolution 450–500</i>	Fig. 1 panel C
<i>mtDNA analysis</i> (IF APPLICABLE)			
Identity	STR analysis	<i>17 sites tested, all matched</i>	<i>Submitted in archive with journal</i> Fig. 1 panel B
Mutation analysis (IF APPLICABLE)	Sequencing	<i>Heterozygous, 36 G₄C₂ repeats</i>	
Microbiology and virology	Mycoplasma	<i>Mycoplasma testing by RT-PCR. Negative.</i>	<i>Supplementary</i>
Differentiation potential	Embryoid body formation	<i>Expression of differentiation markers</i>	Fig. 1 panel H and I
	Teratoma formation	<i>demonstrated through RT-qPCR in embryoid bodies. Ectoderm: SOX1, NESTIN, PAX6, FABP, SLC1A3; Mesoderm: T, EOMES Endoderm: GATA4, FOXA2, SOX17.</i>	
Donor screening (OPTIONAL)	<i>HIV 1 + 2 Hepatitis B, Hepatitis C</i>		
Genotype additional info (OPTIONAL)	<i>Blood group genotyping HLA tissue typing</i>		

have a strong influence in disease onset. Therefore, the possibility of developing cellular models of the disease, directly from the patient's own cells with their genetic background, represents a powerful approach for the development of new therapies.

3. Resource details

Amyotrophic Lateral Sclerosis is a neurodegenerative disease, with upper and lower motor neuron degeneration, whose onset occurs between 40 and 70 years ([Marin et al. 2018](#)). One of the genetic causes associated with this pathology is an expansion of the open reading frame of chromosome 9 (40 % in familial cases) ([Majounie et al. 2012](#)). With less than 24 repeats, the hexanucleotide GGGGCC element isn't pathogenic; the range between 25 and 60 repeats has an uncertain clinical significance, while affected subjects may have thousands of repeated elements ([Gossye et al., 2015](#)).

Here, we collected a skin biopsy from a subject carrying a 36 hexanucleotide expansion (G₄C₂). The presence of these repetitions resulted in not being pathogenic at the time of the biopsy (76 years) because of

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry				
	Antibody	Dilution	Company Cat #	RRID
<i>Pluripotency Markers</i>	Rabbit anti-OCT4	1:100	Life technologies (A13998);	RRID: AB_2534182
	Mouse anti-TRA1-60	1:100	Life technologies (411000)	RRID: AB_2533494
<i>Secondary antibodies</i>	Anti-Rabbit AlexaFluor 488	1:1000	Invitrogen (A11034);	RRID: AB_2576217
	Anti-Mouse AlexaFluor 594	1:1000	Invitrogen (A21422)	RRID: AB_2535844
	Primers Target	Size of band	Forward/Reverse primer (5'-3')	
<i>Episomal Plasmids (qPCR)</i>	eOCT4	83 bp	Fwd: CAT TCA AAC TGA GGT AAG GG	Rev: TAG CGT AAA AGG AGC AAC ATA G
	eKLF4	112 bp	Fwd: CCA CCT CGC CTT ACA CAT GAA GA	Rev: TAG CGT AAA AGG AGC AAC ATA G
	eLIN28	205 bp	Fwd: AGC CAT ATG GTA GCC TCA TGT CCG C	Rev: TAG CGT AAA AGG AGC AAC ATA G
	eL-MYC	80 bp	Fwd: GGC TGA GAA GAG GAT GGC TAC	Rev: TTT GTT TGA CAG GAG CGA CAA T
	eSOX2	66 bp	Fwd: TTC ACA TGT CCC AGC ACT ACC AGA	Rev: TTT GTT TGA CAG GAG CGA CAA T
			Fwd: TTG CTG CAG AAG TGG GTG GA	Rev: TGG CTG ATC TGC TGC AGT GT
<i>Pluripotency Markers (qPCR)</i>	OCT4	179 bp	Fwd: TGA GAG GCG GCC AAA AGG AA	Rev: CAG CGG ACA TGA GGC TAC CA
	LIN28	169 bp	Fwd: GCG AAC CCA AGA CCC AGG CCT GCT CC	Rev: CAG GGG GTC TGC TCG CAC CGT GAT G
	L-MYC	142 bp	Fwd: TTC ACA TGT CCC AGC ACT ACC AGA	Rev: ACC TCA GTT TGA ATG CAT GGG AGA GC
	SOX2	80 bp	Fwd: TCT CAA GGC ACA CCT GCG AA	Rev: CCT GGA AAA TGC TCG GTC GC
<i>Differentiation markers</i>	SOX1		Hs01057642_s1	
	NESTIN		Hs04187831_g1	
	PAX6		Hs00240871_m1	
	T		Hs00610080_m1	
<i>House-Keeping Genes (qPCR)</i>	EOMES		Hs00172872_m1	
	GATA4		Hs00171403_m1	
	FOXA2		Hs00232764_m1	
	SOX17		Hs00751752_s1	
<i>House-Keeping Genes (qPCR)</i>	β-ACTIN	203 bp	Hs99999903_m1	
	β-ACTIN		Fwd: GGC ATCCTC ACC CTGAAG TA	Rev: GGG GTGTTG AAG GTCTCA AA

the lack of neurological disease, even though the daughter of the subject, whose repetitions are increased (56), manifested ALS at the age of 45 (Ruotolo et al. 2024).

To obtain induced pluripotent stem cells (iPSCs), fibroblasts were nucleofected with three non-integrating pCXLE vectors carrying human reprogramming factors (hSOX2, hKLF4, hL-MYC, hLIN28, shp53, hOCT3/4). The first clones were harvested 20 days after nucleofection. Clones that appeared mono-stratified and with well-defined borders (Fig. 1A) were selected for further characterization. First, we ascertained that the selected clones and the starting fibroblasts were from the same cell line by performing a Short Tandem Repeats analysis (STR analysis). Once confirmed, the reprogrammed cells were analyzed to verify that they maintained the correct hexanucleotide expansion (Fig. 1B). We also confirmed that nucleofection did not compromise the karyotypic profile (Fig. 1C) of the cell line. Once the cells reached passage X, we started stemness validation by qPCR analysis (Fig. 1D) and immunofluorescence analysis (Fig. 1E). Real-time PCR analysis showed increased expression of stemness markers (SOX2, OCT4, L-MYC, LIN28, KLF4) in iPSCs compared with fibroblasts, and gene activation was also confirmed by observing OCT4 and TRA1-60 by immunofluorescence. To ensure that the stemness was due to the expression of endogenous genes, we also validated the loss of the exogenous counterpart by qPCR (Fig. 1F). Finally, iPS cells were tested for pluripotency. In vitro analysis was performed by embryoid body formation (Fig. 1G) and validation of pluripotent marker expression by qPCR (Fig. 1H). In addition, as an in vivo analysis, single cells were implanted into immunodeficient mice to perform teratoma formation and subsequent tissue analysis to confirm the presence of all three embryonic layers (Fig. 1I). In this way, we could confirm the success of cell reprogramming (Table 1. Table 2).

4. Materials and methods

4.1. Fibroblast reprogramming

Fibroblasts were cultured at 37 °C, 5 % CO₂ in DMEM-High Glucose with 20 % FetalBovineSerum, 1 % L-Glutammate, 1 % Non-Essential AminoAcids, 1 % Pen/Strept (Sigma-Aldrich). At passage V, 300.000 cells were nucleofected, program FF113, with 1,5 µg of pCXLE-hOCT4-shp53 (Addgene#27077), pCXLE-hSK (Addgene#27078) and pCXLE-hUL (Addgene#27080) at a ratio of 1:1:1. After 7 days, 150.000 cells were plated in a matrigel-coated dish (Corning) in Nutristem-XF (Biological-Industries). Small hiPSCs colonies became visible after 4 weeks from transfection.

4.2. STR characterization

DNA was extracted from both fibroblasts and iPSCs with Dneasy blood and tissue kit (QIAGEN). For STR analysis 17 markers were amplified with PowerPlex® ESX 17 Fast System(Promega). The PCR products were separated with ABI-Prism-3130 DNA-Sequencer and analysed with GeneMapper IDXv3.2 (Applied-Biosystems).

4.3. Karyotype analysis

For karyotype characterization, iPSCs were cultured in flasks for 2–3 days and then treated with a 0.1 µg/mL COLCEMID solution (ThermoFisher-Scientific). Metaphases were obtained by adding a hypotonic solution (30 mM KCl in 10 % foetal bovine serum, followed by incubation at 37 °C for 6 min and fixation with 3:1 ethanol:acetic acid solution. Karyotype analysis was carried out on GTG-banded metaphases (resolution 450–500), on 30 metaphases.

4.4. Hexanucleotide expansion characterization

C9ORF72 expansion was analyzed using AmpliEx-PCR/CE C9ORF72 Kit based on a repeat (G₄C₂)-primed PCR approach.

Products were analyzed in ABI PRISM 3130xl/3500DX Genetic Analyzer (Life-Technologies).

4.5. Immunostaining and imaging

iPSCs (passage XI) were fixed in 4 % Paraformaldehyde for 20 min. Cells were permeabilized and blocked with 0.1 % Triton, 1 % BSA, 10 % NGS. Primary and secondary antibodies were incubated respectively overnight and 1 h, nuclei were labeled with Hoechst (1:10 000). Images were acquired with the Nikon C2 microscope and NisElement 1.49 Program. Scale bars 100 µm.

4.6. RNA extraction and qPCR analysis

Total RNAs were extracted with Trizol reagent (Life-Technologies). RNA was quantified at Qubit 3.0 Fluorometer (Thermo-Scientific), and quality was detected with Agilent 2100 Bioanalyzer. Only RNAs with an RNA Integrity Number ≥ 8 were used for subsequent analysis. Extracted RNAs were digested with DNase I (Life-Technologies), and retro-transcribed with HighCapacity cDNA Reverse-Transcription Kit (Applied-Biosystems).

Real-Time PCR (qRT-PCR) was performed by using SYBR Green PCR Master Mix (Applied-Biosystem) for stemness analysis, and TaqMan Universal PCR Master Mix (Applied-Biosystem) for Pluripotency characterization.

4.7. EBs formation and teratoma assay

iPSCs (passage XII) were grown in floating conditions, switching medium from Nutristem-XF to KOSR medium (DMEM-F12, 20 % Knock-out serum replacement GIBCO, 0.1 mM β-mercaptoethanol, 1xNEAA, 2 mM L-glutamine, 50U/mL Penicillin-Streptomycin), to induce Embryoid Bodies formation. After 14 days pellets were collected.

For teratoma assay, about 3.000.000 cells (passage XI) were injected with 100 µl of Matrigel (Life-Technology) in immunodeficient mice (NOD/SCID). Histological analyses were performed with hematoxylin-eosin imaging. Scale bars 20 µm.

CRedit authorship contribution statement

G. Ruotolo: Data curation. **A. D'Anzi:** Data curation. **A.M.G. Giovenale:** Data curation. **C. Giacometti:** Data curation. **D. Ferrari:** Data curation. **E. Vulcano:** Data curation. **C. D'Asdia:** Data curation. **S. Lattante:** Data curation. **M. Sabatelli:** Conceptualization. **F. Codazzi:** Data curation. **G. Consalez:** Data curation. **M. Marano:** Data curation. **V. Di Lazzaro:** Data curation. **M. Pennuto:** Data curation. **A. Vescovi:** Funding acquisition, Conceptualization. **J. Rosati:** Writing – review & editing, Funding acquisition, Formal analysis, Conceptualization.

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.2024.103540>.

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