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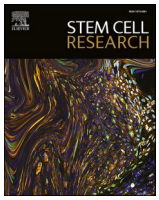

Lab Resource: Single Cell Line

Generation of induced pluripotent stem cells (CSSi017-A)(12862) from an ALS patient carrying a repeat expansion in the C9orf72 gene

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A B S T R A C T

Genetic expansions of the hexanucleotide repeats (GGGGCC) in the C9orf72 gene appear in approximately 40% of patients with familial ALS and 7% of patients with sporadic ALS in the European population, making this mutation one of the most prevalent genetic mutations in ALS. Here, we generated a human induced pluripotent stem cell (hiPSC) line from the dermal fibroblasts of a patient carrying a 56-repeat expansion in an ALS disease-causing allele of C9orf72. These iPSCs showed stable amplification in vitro with normal karyotype and high expression of pluripotent markers and differentiated spontaneously in vivo into three germ layers.

1. Resource table

Unique stem cell line identifier	CSSi017-A (12862) https://hpscreg.eu/cell-line/CSSi017-A
Alternative name(s) of stem cell line	1150 cl 4
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: 45 Sex: female 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)

(continued)

Unique stem cell line identifier	CSSi017-A (12862) https://hpscreg.eu/cell-line/CSSi017-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[56]
Date archived/stock date	1/09/2022
Cell line repository/bank	https://hpscreg.eu/cell-line/CSSi017-A
Ethical approval	Comitato Etico Università Cattolica del Sacro Cuore A.1320/CE/2012

2. Resource utility

The use of primary cells, directly generated from ALS patients with a

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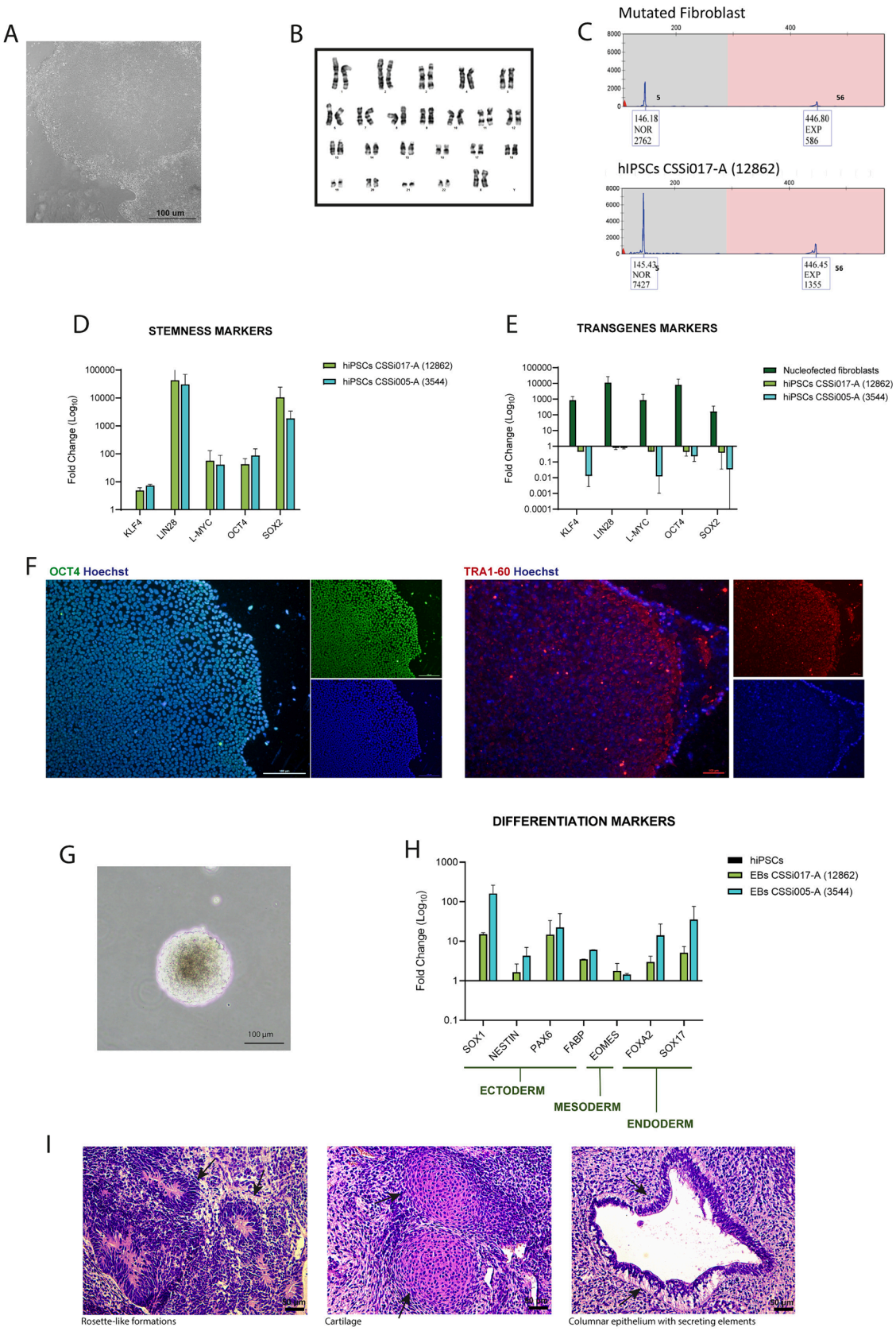
A
100 um
B
C
Mutated Fibroblast
146.18
NOR
2762
446.80
EXP
586
hIPSCs CSSi017-A (12862)
145.43
NOR
7427
446.45
EXP
1355
D
STEMNESS MARKERS
Fold Change (Log10)
hiPSCs CSSi017-A (12862)
hiPSCs CSSi005-A (3544)
KLF4
LIN28
L-MYC
OCT4
SOX2
E
TRANSGENES MARKERS
Nucleofected fibroblasts
hiPSCs CSSi017-A (12862)
hiPSCs CSSi005-A (3544)
F
OCT4 Hoechst
TRA1-60 Hoechst
G
100 µm
H
DIFFERENTIATION MARKERS
hiPSCs
EBs CSSi017-A (12862)
EBs CSSi005-A (3544)
SOX1
NESTIN
PAX6
FABP
EOMES
FOXA2
SOX17
ECTODERM
MESODERM
ENDODERM
I
Rosette-like formations
Cartilage
Columnar epithelium with secreting elements

Fig. 1.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel A
Phenotype	Qualitative analysis Immunocytochemistry	Staining of pluripotency markers: Oct4, Tra 1–60.	Fig. 1 panel F
	Quantitative analysis qRT-PCR	Expression of stemness markers: KLF4, LIN28, L-MYC, OCT4, SOX2.	Fig. 1 panel D
Genotype	Karyotype (G-banding) and resolution	46 XX, Resolution 450–500	Fig. 1 panel B
Identity	STR analysis	17 sites tested, all matched	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	Heterozygous mutation	Fig. 1 panel C
Microbiology and virology	Mycoplasma	Mycoplasma tested by N-Garde Mycoplasma PCR kit. Negative.	Supplementary data. Fig. 1
Differentiation potential	Embryoid body formation Teratoma formation	Genes expressed in embryoid bodies and three embryonic layers in teratoma	Fig. 1 panel H and I
List of recommended germ layer markers	**Expression of these markers must be demonstrated at mRNA (RT PCR) or protein (IF) levels, at least 2 markers need to be shown per germ layer**	Ectoderm: PAX6, SOX1, NESTIN, FABP; Endoderm: SOX17, FOXA2, Mesoderm: EOMES	Fig. 1 panel H
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	

specific mutation, like the expansion in the C9ORF72 gene, is necessary to fully understand, directly in the patient's human cells, which mechanisms are affected by the gene mutation and which therapies can be adopted for treatment.

3. Resource details

Amyotrophic lateral sclerosis, a neurodegenerative disease, is the most common motor neuron disease in adults. The onset of ALS usually occurs in late middle age and leads to fatal degeneration within 3–5 years. Based on the heritability of the disease, approximately 10 % of cases are defined as familial (fALS), whereas 90 % are considered sporadic due to the lack of a family history (sALS). More than 50 genes have been associated to ALS (Wang Hui et al., 2023); of these, the expansion of the hexanucleotide G_4C_2 of the C9ORF72 gene is the most frequent genetic cause in the Caucasian population (Iacoangeli et al., 2019). The pathological behavior of C9ORF72 could depend on either toxicity gain or loss of function, resulting in truncated RNA formation (Chan et al., 2014) and accumulation of dipeptide-repeat (Jiang et al., 2016) in the former case, and haploinsufficiency in the latter (Shi et al., 2018).

We collected a skin biopsy from a 45-year-old ALS patient who was found to carry 56 G_4C_2 repeats in C9orf72. The disease had a very fast progression with death occurring two years after diagnosis. Fibroblasts were reprogrammed into iPSCs by transfecting three pCXLE vectors expressing the human reprogramming factors OCT3/4, shp53, SOX2, KLF4, L- MYC and LIN28 (Okita et al., 2011). After 28 days from nucleofection, the first clones were formed from the reprogramming plate, one of them (Fig. 1A) was manually picked and amplified for characterization. The first analyses we performed were genetic, karyotype analysis confirmed the absence of chromosomal alterations during the reprogramming process (Fig. 1B), and hexanucleotide expansion analysis showed the same GGGCC expansion present in the patient's fibroblasts (Fig. 1C). Furthermore, STR analysis performed on both fibroblasts and iPSCs confirmed the same genetic identity (STR analysis). After ten passages, the iPSC colonies were analyzed by qRT- PCR to assess the expression of endogenous stemness genes (OCT4, SOX2, KLF4, LIN28, MYC) (Fig. 1 D) and silencing of exogenous counterparts (Fig. 1E) with respect to the fibroblast. The gene expression of the reprogrammed line was compared with the published iPSC line CCSi005-A (3544). The stemness of iPSCs was also tested by immunofluorescence analysis, to verify the expression of the intranuclear marker OCT4 and the surface protein TRA 1–60 (Fig. 1F). To confirm the pluripotency of the iPSCs, the iPSCs were treated to form embryoid bodies that were subsequently analyzed, comparing them to the parental iPSCs, for (Fig. 1G) expression of specific genes of the three embryonic layers by qRT-PCR (Fig. 1H). Finally, iPSCs were tested in vivo for their ability to produce teratomas in immunodeficient mice (Fig. 1I). Monthly the iPSC line (pX) was tested to confirm the absence of mycoplasma contamination by using N-Garde Mycoplasma PCR kit (EuroClone) which detects the mycoplasmas in cell culture supernatants by PCR (Supplementary 1).

4. Materials and methods

4.1. Skin biopsy collection and reprogramming

Dermal biopsy was cultured in DMEM–High Glucose, 20 % of FBS, 1 % of L-Glutamine, NEAA and Penicillin-Streptomicin (Sigma Aldrich), at 37 °C and 5 % CO_2. 3×10^5 fibroblasts (passage VI) were nucleofected with 1,5 μg 1:1:1 mix of the episomal plasmids pCXLE-hOCT4-shp53 (Addgene #27077), pCXLE-hUL (Addgene #27080) and pCXLE-hSK (Addgene #27078) with the 4D-Nucleofector® X Unit (Program FF113). After 7 days, $1.5x10^5$ fibroblasts were plated on Matrigel (Corning) coated dish in Nutristem-XF medium (Biological Industries) supplemented with Penicillin-Streptomicin. hiPSCs colonies were mechanically detached and amplified.

4.2. Pluripotency assays

hiPSCs at passage XI were plated in floating condition for the Embryoid Body (EB) formation. Nutristem-XF medium was gradually switched to KOSR medium ((DMEM F12, 20 % Knock-out serum replacement (Gibco), 0.1 mM β-mercaptoethanol, 1X NEAA, 50U/ml Penicillin-Streptomicin, 2 mM L-glutamine) in three days. After 14 days, EB's RNA was extracted and analysed through qRT-PCR. For the in vivo assay, approximately $3x10^6$ cells at passage XIII were enzymatically detached and injected with 100 μl Matrigel (Life Technology) into immune-deficient mice (NOD/SCID). After 1 month, the teratoma was collected for histological analysis.

4.3. Karyotype analysis

For karyotyping, iPSCs at passage VII were cultured in Nutristem medium for 2–3 days. Karyotype analysis was carried out on GTG-banded metaphases (resolution 450–500). Fifteen metaphases were counted and three karyograms were analyzed.

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 AlexaFluor 594	1:1000 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	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	
Pluripotency Markers (qPCR)	Oct-04	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 Rev: CAG CGG ACA TGA GGC TAC CA	
	L-MYC	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 (qPCR)	β-ACTIN	203 bp	Fwd: GGC ATCCTC ACC CTGAAG TA Rev: GGG GTGTTG AAG GTCTCA AA	
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		Hs99999903_m1	

4.4. Hexanucleotide expansion analysis

C9ORF72 was genotyped using AmplideX PCR/CE C9ORF72 Kit based on a repeat (G_4C_2)-primed PCR (RP-PCR) approach. RP-PCR products were analysed in an ABI PRISM 3130xl/3500DX Genetic Analyzer (Life Technologies).

4.5. STR analysis

DNAs of fibroblasts and iPSCs were extracted by Dneasy blood and tissue kit (QIAGEN). 17 distinct STR markers (AMEL, D3S1358, TH01, D21S11, D18S51, D10S1248, D1S1656, D2S1338, D16S539, D22S1045, vWA, D8S1179, FGA, D2S441, D12S391, D19S433, SE33) were amplified through PCR by using the PowerPlex® ESX 17 Fast System (Promega), PCR products were separated on an ABI Prism 3130 DNA sequencer and analyzed by GeneMapper IDX v3.2 (Applied Biosystems).

4.6. Immunofluorescence analysis

iPSCs (pXII) were fixed in 4 % paraformaldehyde for 20 min at room temperature (RT). Permeabilization and blocking were performed with 0.1 % Triton, 1 % BSA, 10 % NGS, for 45 min at RT. Anti-OCT4 (1:100 – Life Technologies) and anti-Tra1-60 (1:100 – Life Technologies) in 5 % BSA were incubated overnight at 4 °C. Anti-Rabbit (AlexaFluor-488 (Invitrogen), anti-Mouse (AlexaFluor-555 (Invitrogen), 1:1000 were incubated for 1 h. Nuclei were labelled with Hoechst 33,342 in 1X PBS (1:10000 – Life Technologies). Images were acquired using a Nikon C2 microscope with 10X objective and NIS Elements 1.49 program.

4.7. Real-time PCR

Total RNAs were extracted with Trizol reagent (Life Technologies). The integrity of RNAs was processed on the Agilent 2100 Bioanalayzer, RNAs with RIN > 8 were *retro*-transcripted using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystem) for the stemness markers and the absence of the exogenous reprogramming factors Table 1, while TaqMan Universal PCR Master Mix (Applied Biosystem) was used for the pluripotency markers (Table 2). Analyses were based on the delta Ct method, and each qRT-PCR analysis was performed in triplicate.

CRediT authorship contribution statement

G. Ruotolo: Writing – original draft, Validation, Data curation. **A. D'Anzi:** Writing – original draft, Validation, Data curation. **A. Casamassa:** Data curation. **M. Mazzoni:** Data curation. **D. Ferrari:** Data curation. **I. Lombardi:** Data curation. **R.M. Carletti:** Data curation. **C. D'Asdia:** Data curation. **I. Torrente:** Data curation. **K. Frezza:** Data curation. **S. Lattante:** Data curation. **M. Sabatelli:** Resources. **M. Pennuto:** Writing – review & editing. **A.L. Vescovi:** Supervision. **J. Rosati:** Writing – review & editing, Writing – original draft, Funding acquisition, Data curation, 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.

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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.2024.103412.

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