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Lab Resource: Stem Cell Line

Generation of induced pluripotent stem cell line, CSSi004-A (2962), from a patient diagnosed with Huntington's disease at the presymptomatic stage

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ABSTRACT

Huntington's disease (HD) is an incurable, autosomal dominant, hereditary neurodegenerative disorder that typically manifests itself in midlife. This pathology is linked to the deregulation of multiple, as yet unknown, cellular processes starting before HD onset. A human iPS cell line was generated from skin fibroblasts of a subject at the presymptomatic life stage, carrying a polyglutamine expansion in *HTT* gene codifying Huntingtin protein. The iPSC line contained the expected CAG expansion, expressed the expected pluripotency markers, displayed in vivo differentiation potential to the three germ layers and had a normal karyotype.

Resource table.

Unique stem cell line identifier	CSSi004-A (2962)
Alternative name(s) of stem cell line	HD256.05 cl1
Institution	Cellular Reprogramming Unit, IRCCS Casa Sollievo della Sofferenza, Viale dei Cappuccini, San Giovanni Rotondo, Foggia Italy
Contact information of distributor	Jessica ROSATI j.rosati@css-mendel.it
Type of cell line	iPSC
Origin	human
Additional origin info	Age: 28 Sex: M Ethnicity: Caucasian
Cell Source	Skin Fibroblasts
Clonality	Clonal
Method of reprogramming	Non integrating episomal vectors
Genetic Modification	NO
Type of Modification	N/A
Associated disease	Huntington Disease
Gene/locus	IT15/HD GENE- 4p16.3
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	June 2017
Cell line repository/bank	N/A
Ethical approval	Casa Sollievo della Sofferenza Ethics Committee, approval number: 75/CE

Resource utility

HD is a completely penetrant genetic disorder, thus the possibility of studying an iPSC line obtained when patient has no evident symptoms of the disease will theoretically allow us to interpret how the pathological process may develop in vitro before clinical manifestations appear, in a pathophysiological human context.

Resource details

Huntington's disease (HD) is a dominantly inherited neurodegenerative disease caused by a polyglutamine expansion in the N-terminus of the huntingtin protein (HTT). Individuals with 35 CAG repeats or fewer are not affected, while those with 36 and more CAG repeats will

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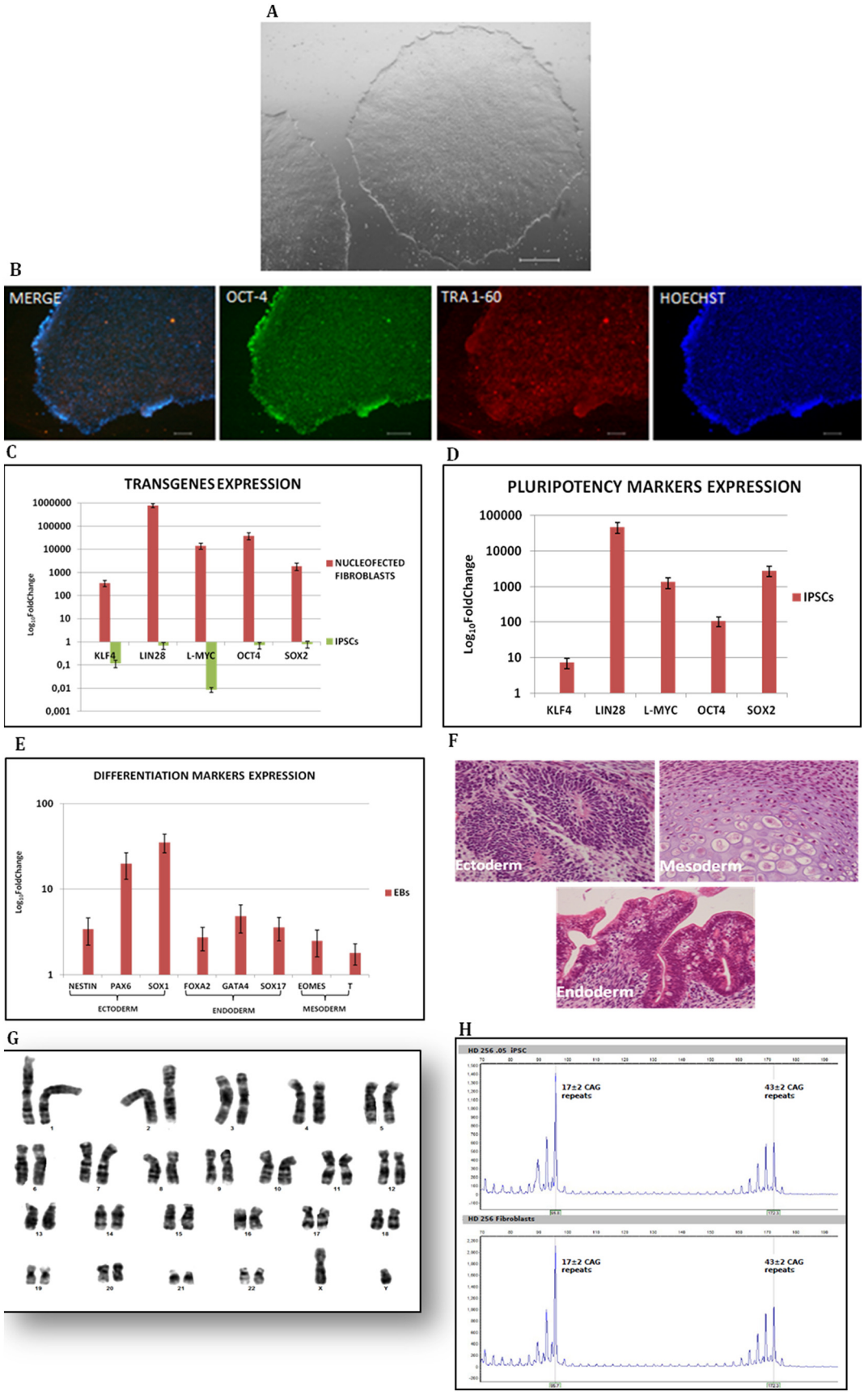
A
B
MERGE
OCT-4
TRA 1-60
HOECHST
C
TRANSGENES EXPRESSION
Log10FoldChange
NUCLEOFECTED FIBROBLASTS
IPSCs
KLF4
LIN28
L-MYC
OCT4
SOX2
D
PLURIPOTENCY MARKERS EXPRESSION
Log10FoldChange
IPSCs
KLF4
LIN28
L-MYC
OCT4
SOX2
E
DIFFERENTIATION MARKERS EXPRESSION
Log10FoldChange
EBs
NESTIN
PAX6
SOX1
FOXA2
GATA4
SOX17
EOMES
T
ECTODERM
ENDODERM
MESODERM
F
Ectoderm
Mesoderm
Endoderm
G
H
HD 256 .05 iPSC
17±2 CAG repeats
43±2 CAG repeats
HD 256 Fibroblasts
17±2 CAG repeats
43±2 CAG repeats

Fig. 1.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal iPSCs morphology	1A
Phenotype	Qualitative analysis	Expression of pluripotency markers: Oct4, Tra1-60	1B
	Quantitative analysis	Expression of pluripotency markers: OCT4, LIN28, L-MYC, KLF4, SOX2	1D
		Silenced transgene expression (from episomes): OCT4, LIN28, L-MYC, KLF4, SOX2	1C
Genotype	Karyotype (G-banding) and resolution	Normal Karyotype: 46 XX, resolution: 450–500	1G
Identity	STR analysis	19 sites tested: all matched	Data available from the authors
Mutation analysis (IF APPLICABLE)	Sequencing	PCR results: 17 ± 1 and 43 ± 2 CAG repeats in fibroblasts 17 ± 1 and 43 ± 2 CAG repeats in IPS cell line.	1H
Microbiology and virology	Mycoplasma	Mycoplasma tested: negative	Supplementary Fig. 1
Differentiation potential	Embryoid body formation and Teratoma formation	Expression of genes of the three germ layers in embryoid bodies: NESTIN, PAX6, SOX1, FOXA2, GATA4, SOX17, EOMES and T. Teratoma: formation of three germ layers.	1E 1F

eventually develop HD at some point in their lives (McColgan and Tabrizi, 2017). The HD mutation is fully penetrant with the time of onset correlating to the number of repeats. Skin fibroblasts were obtained from a patient carrying 43 CAG repeats. This 28-year-old patient was still presymptomatic at the time of the skin biopsy, showing a Diagnostic Confidence Level (DCL) = 0. Skin fibroblasts were reprogrammed into pluripotent stem cells through the nucleofection of episomal vectors containing the Yamanaka factors: OCT4, KLF4, L-MYC, SOX2, LIN28 and sh-p53 (Okita et al. 2011). The established CSSi004-A (2962) iPSC line showed typical human stem cell-like morphology in phase contrast microscopy (Fig. 1A). These iPSC-like colonies were maintained by mechanical splitting in Matrigel coated wells in iPSC media, NutristemXF. Pluripotency of CSSi004-A (2962) line was confirmed by the expression of the transcription factor OCT4 as well as by the expression of the surface marker TRA-1-60 obtained through immunofluorescence analysis (Fig. 1B, Table 1). Episomal plasmids are non-integrative, so they are gradually lost from the cells after rapid cell divisions: after 8 passages, the colonies were free of transgene expression. The RNA from an aliquot of the nucleofected fibroblasts was used as a control of transgene expression (Fig. 1C). A quantitative real-time PCR (qPCR) was used to quantify mRNA expression levels of pluripotent markers in the CSSi004-A (2962) cell line; all five genes, including OCT4, KLF4, LIN28, L-MYC and SOX2 showed high levels of expression in the iPSC line, whereas there was a very low expression in the parental fibroblasts used as controls (Fig. 1D). The CSSi004-A (2962) formed embryoid bodies which spontaneously differentiated into three germ layers; the results were analyzed by qPCR. Ectodermal lineage was confirmed by overexpression of SOX1, NESTIN and PAX6, while the mesodermal lineage was determined through expressions of BRACHIURY (T) and EOMES. Overexpression of GATA4, FOXA2 and SOX17 confirmed the differentiation of the endoderm layer. qPCR reactions were normalized against internal controls (18S) and plotted relative to expression levels of the iPSC (Fig. 1E). Differentiation capacity into three germ layers was also demonstrated by in vivo teratoma formation (Fig. 1F). Authentication of the CSSi004-A (2962) cell line was verified using short tandem repeats analysis and was identical to the parental cell line (STR data available with the authors). Karyotype analysis demonstrated no gain or loss of chromosomes, and no subkaryotypic aberrations (known to be recurrent in human pluripotent stem cells) were detected (Fig. 1G). The allele sizes (17 ± 2 and 43 ± 2 CAG repeats) in fibroblast and IPS cell line were the same (Fig. 1H).

Materials and methods

Skin biopsy and fibroblast reprogramming

The skin biopsy was mechanically dissected and cultured in fibroblast medium (DMEM high glucose, 20% FBS, 2 mM L-glutamine and 1% penicillin–streptomycin (all reagents from Sigma Aldrich)) for 30 days at 37 °C and 5% CO2 to allow fibroblasts to grow out. Briefly, 1 × 10^5 fibroblasts were nucleofected with 3 μg 1:1:1 mix of the episomal plasmids pCXLE-hUL (Addgene #27080), pCXLE-hSK (Addgene #27078) and pCXLE-hOCT4-shp53 (Addgene #27077). The nucleofected cells were plated in fibroblast medium for one week. On day 7 the cells were counted and plated on Matrigel (1:100) (BD

Table 2
Reagents details.

Antibodies used for immunocytochemistry

	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit anti-OCT4	1:100	Life Technologies (A13998)
	Mouse anti-TRA-1-60	1:100	Life Technologies (411000)
Secondary antibodies	anti-Rabbit AlexaFluor 488	1:10000	Invitrogen (A11034)
	anti-Mouse AlexaFluor 555	1:10000	Invitrogen (A21422)

Primers

	Target	Forward/reverse primer (5′–3′)
Episomal genes	eOCT4	Fwd: CAT TCA AAC TGA GGT AAG GG
		Rev.: TAG CGT AAA AGG AGC AAC ATA G
	eKLF4	Fwd: CCA CCT CGC CTT ACA CAT GAA GA
		Rev.: TAG CGT AAA AGG AGC AAC ATA G
	eLIN28	Fwd: AGC CAT ATG GTA GCC TCA TGT CCG C
		Rev.: TAG CGT AAA AGG AGC AAC ATA G
	eL-MYC	Fwd: GGC TGA GAA GAG GAT GGC TAC
		Rev.: TTT GTT TGA CAG GAG CGA CAA T
	eSOX2	Fwd: TTC ACA TGT CCC AGC ACT ACC AGA
		Rev.: TTT GTT TGA CAG GAG CGA CAA T
Pluripotency genes	OCT4	Fwd: CCC CAG GGC CCC ATT TTG GTA CC
		Rev.: ACC TCA GTT TGA ATG CAT GGG AGA GC
	KLF4	Fwd: ACC CAT CCT TCC TGC CCG ATC AGA
		Rev.: TTG GTA ATG GAG CGG CGG GAC TTG
	LIN28	Fwd: AGC CAT ATG GTA GCC TCA TGT CCG C
		Rev.: TCA ATT CTG TGC CTC CGG GAG CAG GGT AGG
	L-MYC	Fwd: GCG AAC CCA AGA CCC AGG CCT GCT CC
		Rev.: CAG GGG GTC TGC TCG CAC CGT GAT G
	SOX2	Fwd: TTC ACA TGT CCC AGC ACT ACC AGA
		Rev.: TCA CAT GTG TGA GAG GGG CAG TGT GC
	18S	Fwd: GGC CCT GTA ATT GGA ATG AGT C
		Rev.: CCA AGA TCC AAC TAC GAG CTT
Differentiation Markers	SOX1	Hs01057642_s1
	NESTIN	Hs04187831_g1
	PAX6	Hs00240871_m1
	EOMES	Hs00172872_m1
	T	Hs00610080_m1
	GATA4	Hs00171403_m1
	FOXA2	Hs00232764_m1
	SOX17	Hs01057642_s1
	18S	Hs03003631_g1

Biosciences). From day 8 on the cells were cultured in NutristemXF medium (Biological Industries). When iPSC colonies reached a sufficient size, they were manually cut and passaged for expansion. Absence of mycoplasma contamination was verified by PCR analysis using EZ-PCR kit (Biological Industries).

Immunofluorescence staining

Cells were fixed using 4% paraformaldehyde for 20 min at room temperature. The cells were incubated with Blocking Buffer (PBS, 20% Normal Goat Serum, 0.1% Triton X-100) for 30 min at room temperature. Next, primary antibodies diluted in blocking buffer were incubated overnight at 4 °C. After washing, Alexa Fluor 594- and/or Alexa Fluor 488-conjugated secondary antibodies in PBS 1× were added 1 h at room temperature. Nuclei were counterstained with DAPI. Micrographs were taken using a Nikon C2 fluorescence microscope and NIS Elements 1.49 software.

Real-time PCR analysis

Total RNA was isolated from cultured cells with Trizol (Life Technologies). Reverse transcription of 1 μg of RNA was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) after digestion with DNAse I (Life Technologies). Each RNA sample was tested in duplicate; 18S was used to normalize transcript abundance and calculations were performed with the delta Ct method. Statistical analyses were performed on three independent experiments. Primers are listed in Table 2.

In vitro spontaneous differentiation

The cell clumps from iPS were plated on Petri dishes in NutristemXF medium. One day later, NutristemXF medium was substituted with differentiation medium: DMEM/F12, 20% KSR (Gibco), 0,1 mM NEAA, 0.1 mM β-mercaptoethanol, 1% Pen/Strep. The embryoid bodies (EBs) were grown for two weeks.

Karyotyping

iPSCs were cultured in chamber slides (Thermo Fisher Scientific) coated with Matrigel in NutristemXF 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. Metaphases were obtained by adding 30 mM KCl in 10%FBS at 37 °C for 6 min and by fixation, using a cold fresh-made 3:1 ethanol:acetic acid solution. Karyotype analysis was carried out on GTG-banded metaphases (450–500 resolution). Fifteen metaphases were counted and three karyotypes analyzed. Only clonal aberrations were considered, following ISCN recommendations.

STR analysis

Fibroblasts and iPSCs DNA was extracted by Dneasy blood and tissue kit (QIAGEN). PCR amplification of 19 distinct STRs (D13S252, D13S305, D13S634, D13S800, D13S628, D18S819, D18S535, D18S978, D18S386, D18S390, D21S11, D21S1437, D21S1409, D21S1442, D21S1435, D21S1446, DXS6803, XHPRT, DXS1187) was carried out using the QST*Rplusv2 kit (Elucigene Diagnostics), PCR products were separated on an ABI Prism 3130 DNA sequencer and analyzed by GeneMapper version 4.0 (Applied Biosystems).

Teratoma formation

Approximately 3×10^6 dispase-treated iPSCs, in 100 μL of Matrigel, were injected into the right flank of nude mice, following ethical guidelines. About 4–6 weeks after injection, tumors were dissected, fixed in 10% formalin (Sigma Aldrich), paraffin-embedded, sectioned and stained with hematoxylin/eosin. The presence of differentiated tissues representative of the three embryonic germ layers was analyzed.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.02.014.

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