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Lab resource: Stem Cell Line

Generation of induced pluripotent stem cell line CSSi008-A (4698) from a patient affected by advanced stage of Dentato-Rubral-Pallidoluysian atrophy (DRPLA)

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ABSTRACT

Dentato-Rubral-pallidoluysian atrophy (DRPLA) is a rare autosomal, dominant, progressive neurodegenerative disease that causes involuntary movements, mental and emotional problems. DRPLA is caused by a mutation in the *ATN1* gene that encodes for an abnormal polyglutamine stretch in the atrophin-1 protein. DRPLA is most common in the Japanese population, where it has an estimated incidence of 2 to 7 per million people. This condition has also been seen in families from North America and Europe. We obtained a reprogrammed iPSC line from a Caucasian patient with a juvenile onset of the disease, carrying 64 CAG repeat expansion in the ATN1 gene.

Resource utility

DRPLA occurs almost only in the Japanese population with a very low frequency in Europe. The iPSC line obtained from a subject with this extremely rare disorder in Caucasian population can become a very valuable tool on understanding the features of disease pathogenesis (Naito et al, 1982).

Resource details

Dentatorubral-pallidoluysian atrophy (DRPLA), one of the spinocerebellar degeneration diseases, is a genetic autosomal dominant condition caused by an expansion of CAG repeats in the coding region of the *ATN1* gene at 12p13.31. The age of onset is inversely correlated with the expanded CAG repeat length. The patients with earlier onset (younger than 20 years old) present a progressive phenotype characterized by intellectual deterioration, behavioral changes, ataxia, myoclonus and epilepsy, whereas the patients with later onset show cerebellar ataxia, choreoathetosis, dementia, and behavioral changes (Burke et al., 1994; Le Ber et al, 2003). Skin fibroblasts were obtained from a 21 years old subject with an early onset of the disease symptoms. He manifested with epilepsy since age 10, ataxia and incoordination since 11 and progressive parkinsonism since 14. One of the alleles of this cell line presented a 63 ± 3 CAG repeat length in the ATN1 gene. After the nucleofection of a mix of pCXLE based episomal vectors carrying the human reprogramming factors (Oct4, Klf4, Sox2 and Myc, Lin28, sh-p53) (Okita et al., 2011), the fibroblasts from the DRPLA subject were reprogrammed into induced pluripotent stem cells. iPSC-like colonies presenting a flat morphology and defined borders (Fig. 1A) were selected and expanded for at least 8 passages before being characterized for their stemness and pluripotency. We used polymerase chain reaction (PCR) to show allele size at the ATN1 CAG $_{(n)}$ repeat region in fibroblast and IPS cell line; the results were 13 ± 1 and 63 ± 3 CAG repeats in fibroblast and 13 ± 1 and 65 ± 3 CAG repeats in IPS cell lines (Fig. 1B). iPSC line showed a normal karyotype (Fig. 1C). Short tandem repeats analysis showed that the reprogrammed cell line was identical to the parental fibroblasts (STR data not shown). Immunostaining analysis against Oct4 and Tra-1-60 demonstrated the expression of both pluripotency markers (Fig. 1D). qReal-Time PCR analysis confirmed a higher expression of several endogenous pluripotency markers (OCT4, KLF4, LIN28, SOX2 and MYC) in iPSC line compared to the un-nuclefected fibroblasts, eight splittings after the

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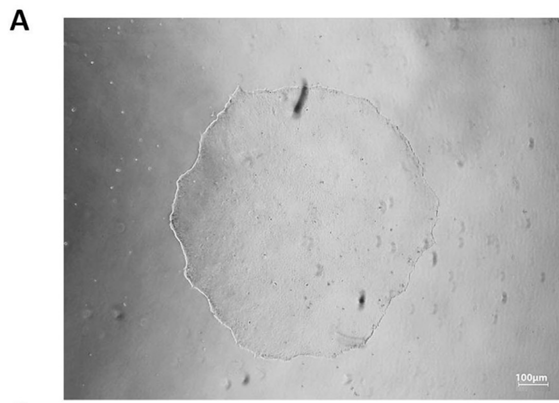
A



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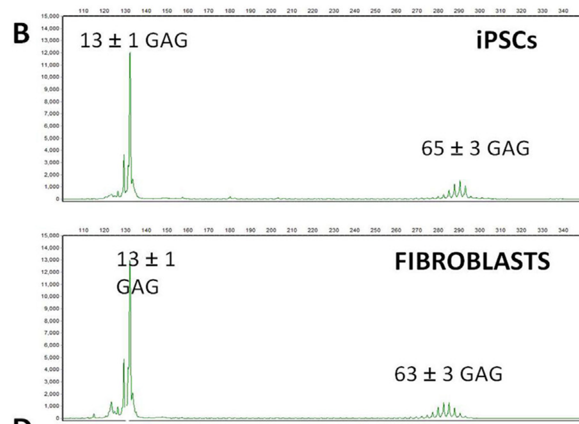

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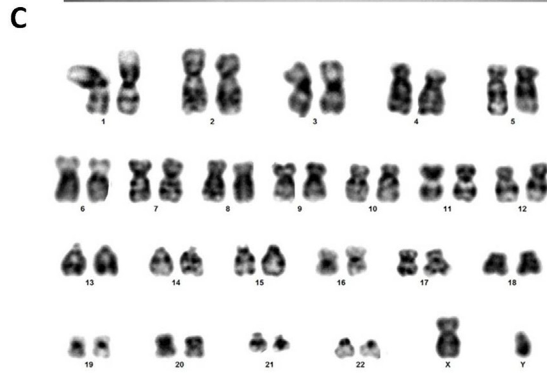

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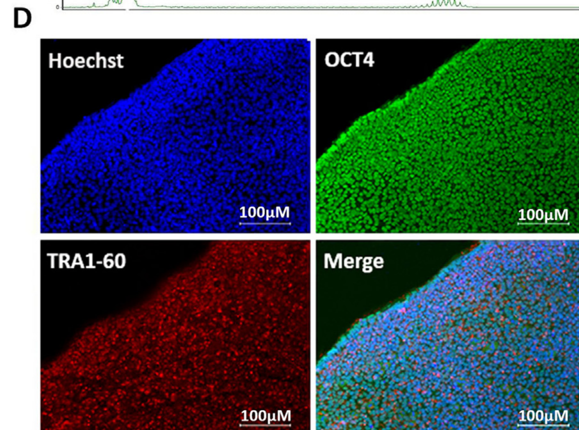

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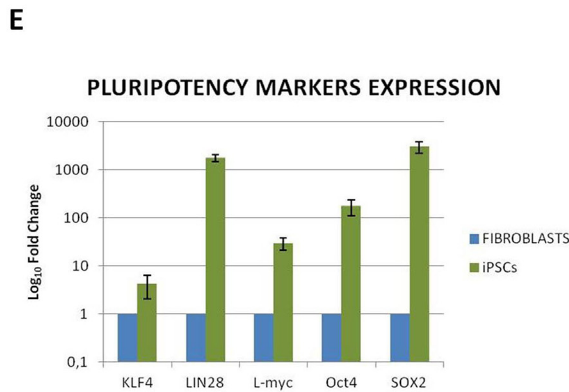

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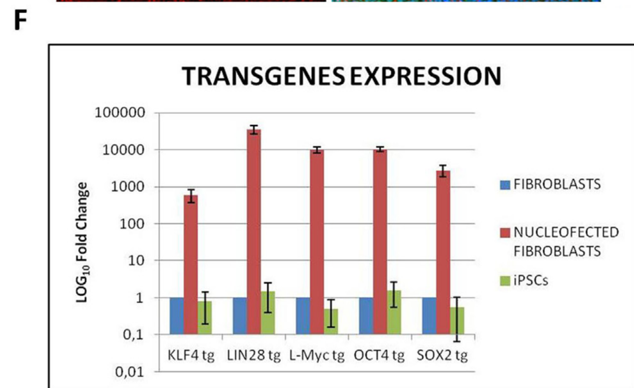

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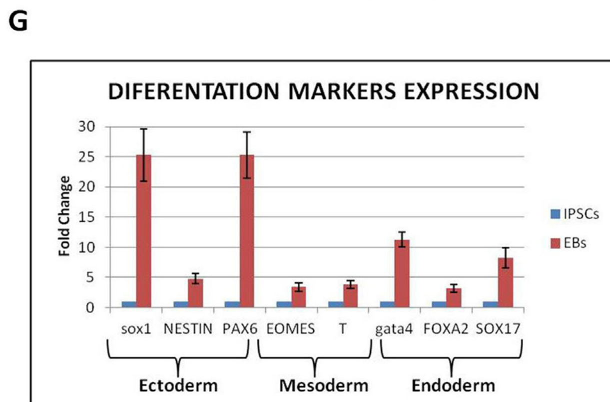

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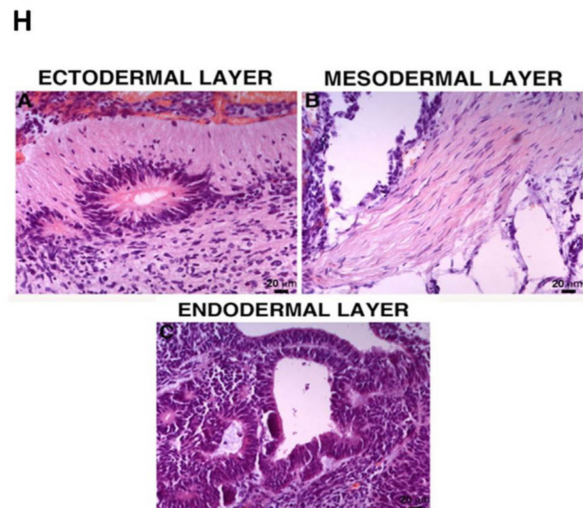

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Fig. 1. A. Image of iPS acquired by optical microscope, scale bar = 100 µm; **B.** Polymerase Chain reaction (PCR) results showing CAG $_{(n)}$ repeat region of the *ATN1* gene in fibroblast and IPS cell line. **C.** Representative karyotype of iPS cells; **D.** Immunofluorescence analysis of pluripotency markers such as OCT4 and TRA 1-60 in iPSC, scale bar = 100 µm; **E.** qRT-PCR analysis of pluripotency markers, using 18S as reference; **F.** qRT-PCR analysis of transgene expression in iPSC and nucleofected fibroblasts used as control of transgene expression; **G.** qRT-PCR analysis of differentiation markers, using 18S as reference; **H.** Histological analysis of teratoma produced by iPSC.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	The cell line shows a normal iPSCs morphology visually	*1A*
Phenotype	Immunocytochemistry	The cell line shows expression for pluripotency markers: Oct4, Tra1–60	*1D*
	qRT-PCR	The cell line shows expression for pluripotency markers: *OCT4, LIN28, L-MYC, KLF4, SOX2.* *Silenced transgene expression (from episomes): OCT4, LIN28, L- MYC, KLF4, SOX2*	*1E, 1F*
Genotype	Karyotype (G-banding) and resolution[	E.g. *46XY, Resolution 450–500*	*1C*
Identity	Microsatellite PCR	*N/A*	*N/A*
	STR analysis	*Tested 19 sites: all matched*	*Data is available from the authors*
Mutation analysis (IF APPLICABLE)	qPCR	*CAG repeat number in ATN1 gene performed by PCR: 13 ± 1 and 63 ± 3 CAG repeats in fibroblasts 13 ± 1 and 65 ± 3 CAG repeats in iPS cell line.*	*1B*
	Southern Blot OR WGS	*N/A*	*N/A*
Microbiology and virology	Mycoplasma	*Mycoplasma tested: negative*	Suppl. Fig. 1
Differentiation potential	Embryoid body formation (qPCR) Teratoma formation	Genes expressed in embryoid bodies: SOX1, NESTIN, PAX6, EOMES, GATA4 and FOXA2 Proof of three germ layers formation	*1G;1H*
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	*N/A*	*N/A*
Genotype additional info (OPTIONAL)	Blood group genotyping	*N/A*	*N/A*
	HLA tissue typing	*N/A*	*N/A*

first isolation of the single clone (Fig. 1E), and confirmed the absence of the expression of the nucleofected transgenes (Fig. 1F). When cultured in the absence of anti-differentiation factors, iPSCs spontaneously aggregate to form embryoid bodies, as confirmed by qRT-PCR showing the induction of ectodermal markers (SOX1, Nestin and PAX6), mesodermal markers (Brachiury (T) and Eomes) and endodermal markers (GATA4, FOXA2 and SOX17)(Fig. 1G). When injected subcutaneously in immunocompromised mice iPSC's were able to generate teratomas comprising tissue representatives of all three embryonic germ layers (ectoderm, mesoderm and endoderm) (Fig. 1H), thus attesting to their differentiation potential. (See Table 1.)

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 (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 Biosciences). From day 8 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 in 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. Fifteen metaphases were

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	*Rabbit anti-OCT4*	*1:100*	*Life Technologies (Cat# A13998 RRID AB_2534182)*
	Mouse anti-TRA-1-60	*1:100*	*Life Technologies (Cat# 411000 RRID AB_2533494)*
Secondary antibodies	*Anti-rabbit AlexaFluor 488*	*1:1000*	*Invitrogen (Cat# A11034 RRID AB_2576217)*
	anti-Mouse AlexaFluor 555	*1:10000*	*Invitrogen (Cat# A21422 RRID AB_2535844)*

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 CAGGGT 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	*Hs00751752_s1*
	18S	*Hs03003631_g1*

counted and three karyotypes analyzed. Only clonal aberrations were considered, following ISCN recommendations.

Mutation analysis

Determination of the CAG repeat number in *ATN1* gene was performed by PCR using published primer (Majounie et al., 2007) followed by fragment sizing through capillary electrophoresis adopting on ABI3130 detection system (ThermoFisher) and GeneMarker software (SoftGenetics). The accuracy of CAG repeats was established in accordance with the ENQN (European Molecular Genetics Quality Network) which reports as "acceptable" an error $\pm$ 1 repeat for allele ≤ 42 and $\pm$ 3 repeats for allele > 42 (Losekoot et al., 2013

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 3×10^5 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.

Key resources table

Unique stem cell line identifier	CSSi008-A (4698)
Alternative name(s) of stem cell line	*AT214–01-02*
Institution	*Cellular Reprogramming Unit, IRCCS Casa Sollievo della Sofferenza, Viale dei Cappuccini, San Giovanni Rotondo, Foggia Italy*
Contact information of distributor	*Jessica ROSATIj.rosati@css-mendel.it*
Type of cell line	*iPSC*
Origin	*human*
Additional origin info	*Age: 21* *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	Dentatorubral-pallidoluysian atrophy (DRPLA)
Gene/locus	*ATN1/DRPLA GENE*- 12p13.31
Method of modification	*N/A*
Name of transgene or resistance	*N/A*
Inducible/constitutive system	*N/A*
Date archived/stock date	*June 2018*
Cell line repository/bank	*N/A*
Ethical approval	*Casa Sollievo della Sofferenza Ethic Committee, approval number: 75/CE*

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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.2019.101551.

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