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Lab Resource: Stem Cell Line

Production and characterization of human induced pluripotent stem cells (iPSCs) from Joubert Syndrome: CSSi001-A (2850)

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ABSTRACT

Joubert Syndrome (JS) is a rare autosomal recessive or X-linked condition characterized by a peculiar cerebellar malformation, known as the molar tooth sign (MTS), associated with other neurological phenotypes and multiorgan involvement. JS is a ciliopathy, a spectrum of disorders whose causative genes encode proteins involved in the primary cilium apparatus. In order to elucidate ciliopathy-associated molecular mechanisms, human induced pluripotent stem cells (hiPSCs) were derived from a patient affected by JS carrying a homozygous missense mutation in the AHI1 gene (p.H896R) that encodes a protein named Jouberin.

Resource table

Unique stem cell line identifier	CSSi001-A (2850)
Alternative name(s) of stem cell line	Joub03 cl2, COR419
Institution	Casa Sollievo della Sofferenza - Viale dei Cappuccini, 71013 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: 40 yrs. Sex: Male Ethnicity: Caucasian/Italian
Cell source	Dermal Fibroblasts
Clonality	Clonal
Method of reprogramming	Non integrating episomal vectors
Genetic modification	NO
Type of modification	N/A
Associated disease	Joubert Syndrome
Gene/locus	(AHI1):c.2687A > Gp
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	October 2016
Cell line repository/bank	N/A
Ethical approval	Casa Sollievo della Sofferenza Ethics Committee, approval number: 2292/DS

Resource utility

JS is characterized by phenotypic variability and genetic heterogeneity that complicates the comprehension of molecular mechanisms at the basis of disease. Established JS patient's iPSCs, which can be a resource of patient-specific neurons, will be a powerful tool to elucidate this neurodevelopmental disorder.

Resource details

Joubert Syndrome (JS) is a recessive or X-linked disorder characterized by hypotonia, ataxia, cognitive impairment and a distinctive midhindbrain malformation (Joubert et al., 1969). It is part of a larger spectrum of developmental disorders commonly known as ciliopathies because phenotypic traits are caused by defects in primary cilia biogenesis and function (Valente et al., 2014). Primary cilia are microtubule-based structures projecting from the cell surface and serve as a

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focused center for signal transduction. They mediate key pathways of embryonic development such as Wnt and Shh signalling. To date the role of cilia in regulating canonical Wnt remains unclear. AHI1 gene, mutated in the homozygous status in the present patient, encodes a multidomain protein also known as Jouberin, which consists of an N-terminal coiled-coil domain, seven WD40 repeats, and a C-terminal SH3 domain (Ferland et al., 2004; Valente et al., 2006). Jouberin localizes to the basal body of primary cilia and its function is required for ciliogenesis in cultured cells. Published data correlate AHI1 mutations with a deregulation of the Wnt pathway (Lancaster et al., 2011). In this study, skin fibroblasts from a 40-years-old man were reprogrammed into iPSCs using non-integrative episomal vectors

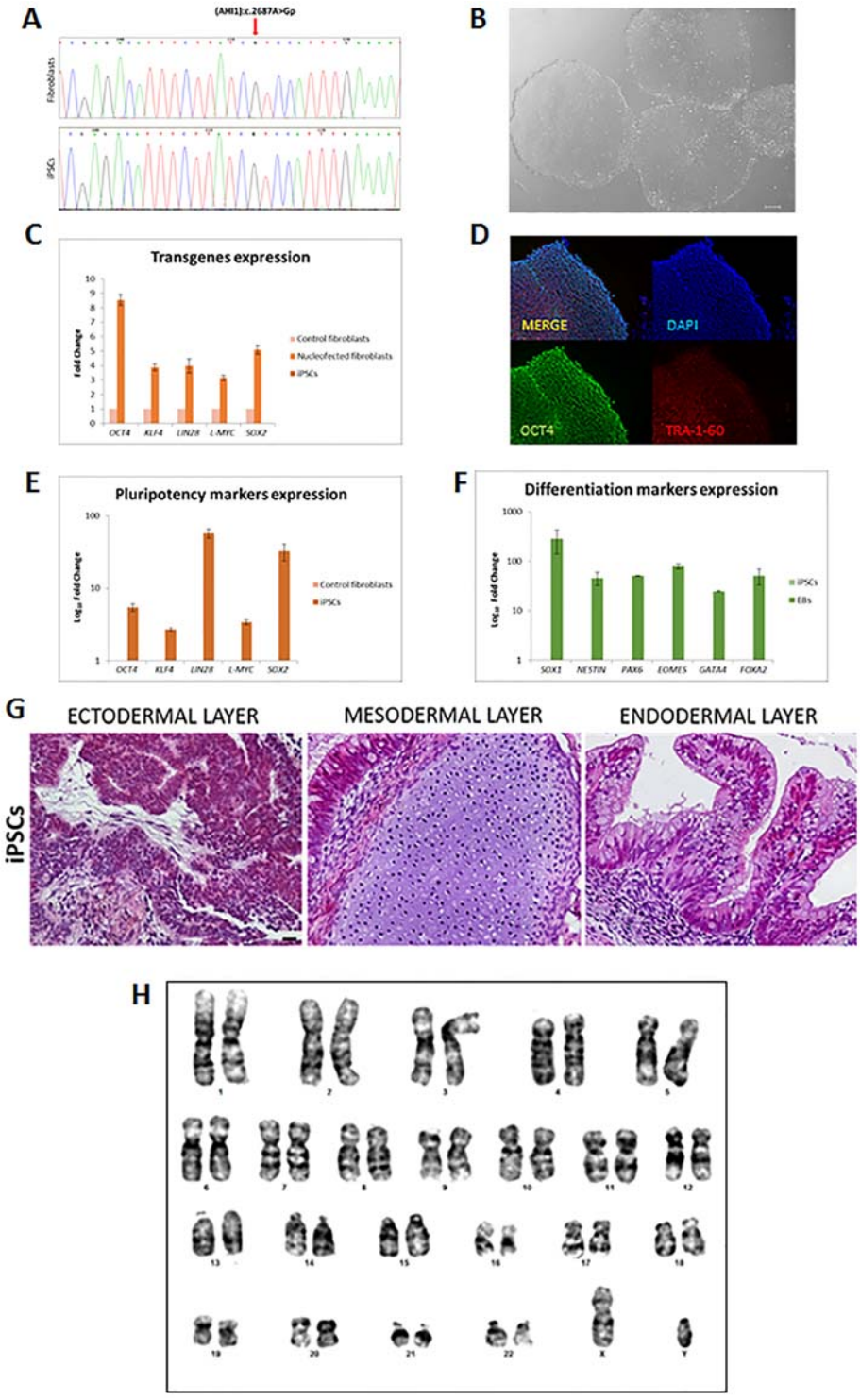

Fig. 1. A. Genomic sequence of mutation of the *AHI1* gene in JS fibroblasts (upper panel) and induced pluripotent stem cells (lower panel) showing a c.2687A > Gp substitution in homozygous marked with a red arrow. **B.** JS iPSC colonies morphology. Scale bars correspond to 100 μm **C.** Transgenes expression analysis through qRT-PCR: nucleofected fibroblasts are used as positive control. Data is shown as the fold change ($2^{-\Delta\Delta Ct}$). **D.** Immunofluorescence staining of JS iPSC with the stemness marker OCT3/4 (green) and the endogenous surface marker TRA-1-60 (red). Cell nuclei were stained with DAPI (blue) and the three channels were merged. Scale bars correspond to 100 μm. **E.** Pluripotent expression analysis through qRT-PCR. Relative expression is shown as the $\log_{10}$ fold change ($2^{-\Delta\Delta Ct}$) with *18S* and fibroblasts as references. **F.** Expression analysis of three germ layers markers. Relative expression is shown as the $\log_{10}$ fold change ($2^{-\Delta\Delta Ct}$) with *18S* and iPSC as references. **G.** Teratoma from the JS iPSC showing tissues from all three germ layers. **H.** Karyotype analysis.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1B
Phenotype	Immunocytochemistry	Staining of pluripotency markers: OCT4; TRA-1-60	Fig. 1C–E
	qRT-PCR	Expression of pluripotency markers: OCT4, LIN28, L-MYC, KLF4, SOX2	
Genotype	Karyotype (G-banding) and resolution	46 XY, Resolution 450–500	Fig. 1H
Identity	STR analysis	19 distinct loci: all matched	STR available with the author
Mutation analysis	Sequencing	Homozygous mutation	Fig. 1A
Microbiology and virology	Mycoplasma	Mycoplasma tested by N-Garde Mycoplasma PCR kit (EuroClone): Negative	Supplementary Fig. 1
Differentiation potential	Embryoid bodies formation and Teratoma formation	Genes expressed in embryoid bodies: SOX1, NESTIN, PAX6, EOMES, GATA4 and FOXA2 Proof of three germ layers formation	Fig. 1G, F

containing the reprogramming factors OCT4, SOX2, L-MYC, KLF4, LIN28, shp53. Genomic DNA sequencing verified that the disease-related mutation (AHI1): c.2687A > G was retained in the generated iPSCs (Fig. 1A). JS iPS cell colonies displayed a typical ES-like colony morphology and growth behavior (Fig. 1B). We confirmed the absence of the exogenous reprogramming factor genes by qPCR after ten passages using, as positive control, the fibroblasts after one week from episomal nucleofection (Fig. 1C). Immunofluorescence analysis revealed expression of transcription factor OCT4 and surface marker TRA-1-60, characteristics of pluripotent stem cells (Fig. 1D). OCT4 protein is correctly shown in the nuclear compartment of the cells, while additional TRA-1-60 marker is properly present on cell surface. The endogenous expression of the pluripotency markers LIN28, OCT4, KLF4, SOX2, L-MYC was evaluated by qPCR which indicated a strong upregulation of these markers in the iPSCs relative to the fibroblast controls (Fig. 1E). JS iPSCs were differentiated both *in vitro* through embryoid bodies formation and *in vivo* through a subcutaneous transplant in nude mice. The expression of three germ layers markers was demonstrated through qPCR (Fig. 1F). Teratoma assay showed the presence of normal differentiation towards endodermal, ectodermal and mesodermal layers (Fig. 1G). JS iPS cell line was negative for Mycoplasma contamination (Supplementary Fig. 1). The JS iPS cell line displayed a normal karyotype (46, XY) after more than twenty culture passages (Fig. 1H). Short tandem repeat (STR) analysis showed an identical DNA profile between the donor's fibroblasts and the derived iPSCs (STR data is available with the authors). Taken together, we have successfully reprogrammed

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)

SYBR green primers used for qPCR	Target	Forward/reverse sequence (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 markers	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
House-keeping gene	18S	Fwd: GGCCCTGTAATTGGAATGAGTC
		Rev: CCAAGATCCAACTACGAGCTT

TaqMan primers used for qPCR	Target	Probe
Differentation markers	SOX1	Hs01057642_s1
	NESTIN	Hs04187831_g1
	PAX6	Hs00240871_m1
	EOMES	Hs00172872_m1
	GATA4	Hs00171403_m1
	FOXA2	Hs00232764_m1
	18S	Hs03003631_g1

JS dermal fibroblasts into iPSCs that can be used to generate neural cells in order to study the pathogenic mechanism underlying Joubert Syndrome caused by mutation in the AHI1 gene.

Materials and methods

Cellular reprogramming

Human fibroblasts were nucleofected using the episomal vectors expressing OCT4, KLF4, SOX2, L-MYC, LIN-28, sh-p53 (Addgene) and cultured for 6 days in fibroblast medium before being plated on Matrigel (Corning Inc., USA, 1:100) plates. The day after, the fibroblast medium was replaced with the NutristemXF iPS medium, (Biological Industries). The emergent hiPSC colonies were picked according to their hESC-like colony morphology and expanded under feeder-free conditions. Absence of mycoplasma contamination was verified by PCR analysis using N-Garde Mycoplasma PCR kit (EuroClone) (Table 1).

qPCR analysis

Total RNAs were extracted using Trizol reagent (Life Technology) and cDNA synthesized using the High capacity cDNA RT (Life Technology). qPCR analysis was performed in three independent biological experiments with SYBR green primers (Table 2) for pluripotency markers and TaqMan (Thermo Fisher Scientific) primers (Table 2) for three germ layers. The expression ratio of the target genes was calculated by using the $2^{-\Delta\Delta Ct}$ method, considering 18S as reference gene.

Embryoid body formation

For the generation of EBs, cells were resuspended in DMEM/F12 medium supplemented with 20% KSR, 0.1 mM NEAA, 1 mM L-Glutamine, 50 μM 2-mercaptoethanol, 50 U/mL penicillin and 50 mg/mL streptomycin. Fourteen days later, EBs were pelletted and RNAs were extracted for qRT-PCR analysis.

Immunofluorescence staining

Cells were fixed using 4% paraformaldehyde and stained. 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, listed in Table 2, diluted in blocking buffer were added and incubated overnight at 4 °C. After washing, Alexa Fluor 594- and/or Alexa Fluor 488-conjugated secondary antibodies were added 1 h at room temperature. Nuclei were counterstained with DAPI. Microphotograps were taken using a Nikon C2 fluorescence microscope and NIS Elements 1.49 software.

Teratoma formation

Approximately 3×10^6 dispase-treated iPSCs, in 100 μl of Matrigel, were injected into the right flank of nude mice. After 1 month, tumors were collected for histological analysis to check for their *in vivo* differentiation capacity into derivatives of all three germ layers.

Karyotyping

iPSCs were cultured in chamber slides (Thermo Fisher Scientific) coated with Matrigel (1:100) in Nutristem medium for 2–3 days. Cells were treated with a COLCEMID solution (Thermo Fisher Scientific) with a final dilution of 0.1 μg/mL for 60 min at 37 °C. Metaphases were obtained by adding a hypotonic solution (30 mM KCl in 10%FBS) followed by incubation at 37 °C for 6 min and by fixation using cold fresh-made 3:1 ethanol:acetic acid solution. Karyotype analysis was carried out on GTG-banded metaphases (Resolution 450–500). Fifteen metaphases were counted and three karyotypes analyzed. Only clonal aberration were considered: the same structural alteration, the gain of a particular chromosome or the loss of a whole chromosome had to be present in at least two or three different cell colonies, respectively, following the ISCN recommendations.

STR analysis

Fibroblasts and iPSCs DNA was extracted by Dneasy blood and tissue kit (QIAGEN) following manufacturer's suggestions. 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).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.01.012.

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