Stem Cell Research 28 (2018) 153–156

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Lab Resource: Stem Cell Line

Production and characterization of CSSI003 (2961) human induced pluripotent stem cells (iPSCs) carrying a novel puntiform mutation in RAI1 gene, Causative of Smith–Magenis syndrome

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ARTICLE INFO

Article history:
Received 25 January 2018
Received in revised form 14 February 2018
Accepted 20 February 2018
Available online 21 February 2018

ABSTRACT

Smith-Magenis syndrome (SMS) is a complex genetic disorder characterized by developmental delay, behavioural problems and circadian rhythm dysregulation. About 90% of SMS cases are due to a 17p11.2 deletion containing retinoic acid induced1 (RAI1) gene, 10% are due to heterozygous mutations affecting RAI1 coding region. Little is known about RAI1 role.

Resource table.

Unique stem cell line identifier	*CSSi003-A (2961)*
Alternative name(s) of stem cell line	*COL04 clE2*
Institution	Cellular Reprogramming Unit, IRCCS 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:37* *Sex: Female* *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	*Smith-Magenis syndrome*
Gene/locus	*NM_030665.3:c.1194delC*
Method of modification	*N/A*
Name of transgene or resistance	*N/A*
Inducible/constitutive system	*N/A*
Date archived/stock date	*April 2017*
Cell line repository/bank	*N/A*
Ethical approval	*Casa Sollievo della Sofferenza Ethical Committee, approval number: 75/CE*

Resource utility

Smith-Magenis is a syndrome impacting craniofacial features, physical/mental development, cognition and behavior. RAI1 protein is highly expressed in the brain, suggesting a central role in brain function. The production of iPSC carrying a RAI1 haploinsufficiency will allow us to study the mutated gene's pathological influence on brain development and function.

Resource details

The hallmarks of Smith-Magenis syndrome (OMIM # 182290) are sleep disturbances, multiple developmental anomalies,

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https://doi.org/10.1016/j.scr.2018.02.016

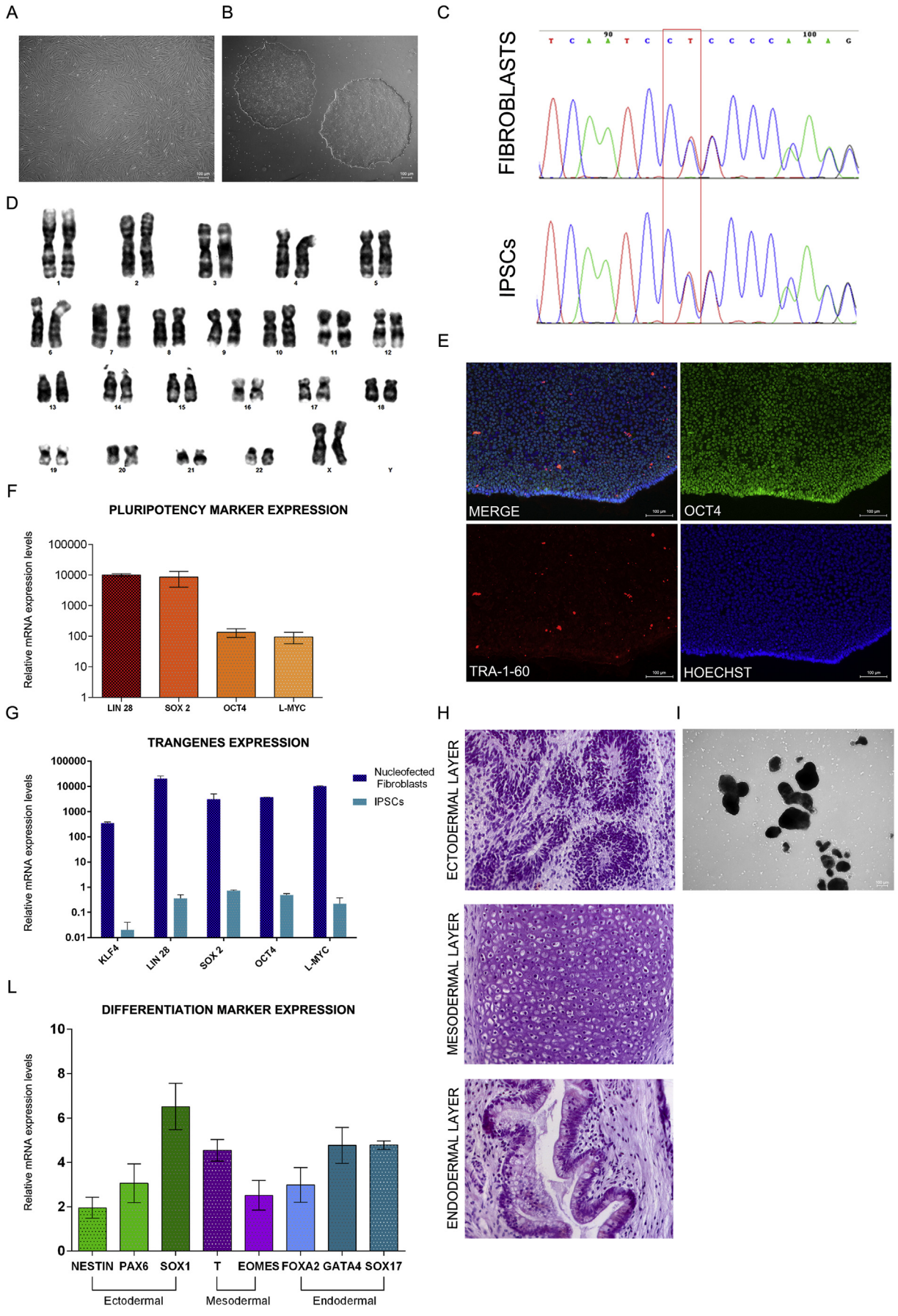
A
B
C
FIBROBLASTS
IPSCs
D
E
MERGE
OCT4
TRA-1-60
HOECHST
F
PLURIPOTENCY MARKER EXPRESSION
Relative mRNA expression levels
LIN 28
SOX 2
OCT4
L-MYC
G
TRANGENES EXPRESSION
Nucleofected Fibroblasts
IPSCs
KLF4
LIN 28
SOX 2
OCT4
L-MYC
H
I
ECTODERMAL LAYER
MESODERMAL LAYER
ENDODERMAL LAYER
L
DIFFERENTIATION MARKER EXPRESSION
NESTIN
PAX6
SOX1
T
EOMES
FOXA2
GATA4
SOX17
Ectodermal
Mesodermal
Endodermal

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	*Normal*	Fig. 1 *panel B*
Phenotype	Immunocytochemisty	*Staining of pluripotency markers: Oct4; Tra-1-60.*	Fig. 1 *panel E, F*
	qRT-PCR	*Expression of pluripotency markers: OCT4, LIN28, L-MYC, KLF4, SOX2*	
	Flow cytometry	*N/A*	
Genotype	Karyotype (G-banding) and resolution	*46 XX,* *Resolution 450–500*	Fig. 1 *panel D*
Identity	Microsatellite PCR (mPCR)	*N/A*	
	STR analysis	*19 sites (D13S252, D13S305, D13S634, D13S800, D13S628, D18S819, D18S535, D18S978, D18S386, D18S390, D21S11, D21S1437, D21S1409, D21S1442, D21S1435, D21S1446, DXS6803, XHPRT, DXS1187): all matched*	*Data available with the authors*
Mutation analysis (if applicable)	Sequencing	*heterozygous NM_030665.3:c.1194del*	Fig. 1 *panel C*
	Southern Blot OR WGS	*N/A*	
Microbiology and virology	Mycoplasma	*Mycoplasma tested by* N-Garde Mycoplasma PCR kit (EuroClone)*: Negative*	*Supplementary Fig. 1*
Differentiation potential	In vitro: embryoid body formation	*Genes expressed in embryod bodies: SOX1, NESTIN, PAX6, EOMES, T, GATA4, FOXA2, SOX 17.*	Fig. 1 *panel I*
	In vivo: teratoma formation	*Proof of three germ layers formation.*	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*	

psychopathological behavior, severe cognitive impairment and obesity (Chen et al., 2015). The incidence of SMS is estimated at 1:25000 births, although this value may be underreported, and no gender differences have been observed (Elsea and Girirajan, 2008). *RAI1* gene, the causative gene of SMS, contains six exons which generate an ~8.5-kb mRNA and a 1906-amino-acid nuclear protein (Carmona-Mora et al., 2012). Rai1 protein has transcriptional factor activity, however little is known about its role. We collected skin fibroblasts (Fig. 1A) from a patient carrying the novel mutation NM_030665.3:c.1194delC, which generates a truncated form of RAI1 protein. We reprogrammed SMS fibroblasts into iPSCs, using non-integrative episomal vectors containing the reprogramming factors OCT4, SOX2, L-MYC, KLF4, LIN28, shp53. We characterized the clones with ES-like morphology (Fig. 1B). Genomic DNA sequencing has been performed and we have verified that the disease-related mutation (NM_030665.3:c.1194delC) was retained in the iPSCs (Fig. 1C). Moreover, we confirmed that, after more than twenty passages, the SMS iPSCs line displayed a normal karyotype (46, XX) (Fig. 1D). We analyzed the expression of the surface marker TRA-1-60 and the transcription factor OCT4 through immunofluorescence analysis (Fig. 1E): TRA-1-60 protein is present in cell surfaces and OCT4 protein is evident in the nuclear compartment of the iPS cells. We also verified the presence of endogenous expression of the pluripotency markers, *LIN28*, *SOX2*, *OCT4* and *L-MYC* using qPCR (Fig. 1F). After ten passages of amplification, we confirmed the suppression of exogenous reprogramming factor expression to negligible levels by qPCR using primers against episomal sequences flanking the exogenous genes; the positive control is constituted by fibroblasts collected at 24 h from episomal nucleofection (Fig. 1G). Pluripotency capacity of the SMS iPSCs was tested differentiating the iPSC both in vitro through embryoid bodies assay (Fig. 1I) and in vivo through a teratoma assay. All three germ layers: ectodermal, mesodermal and endodermal were present in the teratoma, as demonstrated by immune-histochemical analysis (Fig. 1H). We demonstrated a high expression of the markers of each germ layer through qPCR (Fig. 1L). Short tandem repeats analysis (STR) showed that the DNA profile of the donor's fibroblasts was identical to the derived iPSCs (data available with the authors). All cells tested negative for Mycoplasma contamination (Supplementary file 1). These data demonstrate that we have successfully generated a stable SMS patient specific iPSCs line useful for studying the Smith-Magenis disorder, at the molecular level (Table 1).

Materials and methods

Skin biopsy and fibroblast reprogramming

Skin fragments were cut into small fragments and plated on a tissue-culture dish in Dulbecco's Modified Eagle Medium (DMEM) High Glucose supplemented with 20% FBS, 2 mM L-Glutamine, 100 U/ml Penicillin-Streptomicin and 1× Non-Essential Amino Acids (Sigma Aldrich) and cultured for 30 days to allow fibroblasts to grow.

Approximately 1×10^5 fibroblasts were nucleofected with a mix of three pCXLE-based episomal vectors: pCXLE-hOCT4-shp53, pCXLE-hUL and pCXLE-hSK (Addgene). After nucleofection, cells were cultured for 6 days in fibroblasts medium. On day 7, cells were plated on Matrigel (Corning) coated plates in NutristemXF medium (Biological Industries). The emergent hiPSC colonies were expanded under feeder-free conditions. Absence of mycoplasma contamination was verified using N-Garde Mycoplasma PCR kit (EuroClone).

q-PCR

Total RNAs were isolated using TRIzol reagent (Life Technologies). cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed using SYBR Green primers for pluripotency markers and TaqMan primers for differentiation markers (Table 2). Each reaction was performed in triplicate with *β-ACTIN* gene as reference. $2^{-\Delta\Delta CT}$ method was adopted to calculate the relative expression levels.

Fig. 1. Characterization of SMS iPSC. A. Smith-Magenis patient-derived skin fibroblast cell line. Scale bar, 100 μm. B. iPSC colonies morphology. Scale bar, 100 μm. **C.** Sequencing chromatograms of mutated *RAI1* gene in SMS fibroblasts (upper panel) and SMS iPSC (lower panel): the box indicates the mutation site. D. normal iPSC karyogram. E. Immunofluorescence staining shows expression of pluripotency markers OCT3/4 (green) and TRA-1-60 (red) in SMS iPSC. Cell nuclei were stained with HOECHST (blue). Scale bar, 100 μm. F. qRT-PCR analysis for pluripotent markers. The experiment was repeated twice in triplicate using three cDNAs. G. qRT-PCR analysis for transgenes transcripts in SMS iPSC. This experiment was repeated twice in triplicate using different cDNAs. H. Histological sections of teratoma derived from SMS iPSC, stained with hematoxylin and eosin. I. COL04 EBs in suspension. Scale bar, 100 μm. L. qRT-PCR analyses of all three germ layer markers from SMS embryoid bodies. This experiment was repeated twice in triplicate using independently prepared cDNAs.

Table 2
Antibodies used for immunocytochemistry/flow-cytometry.

	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′)
Exogenous 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
	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	*β-ACTIN*	*Fwd: GGC ATC CTC ACC CTG AAG TA*
		Rev: GGG GTG TTG AAG GTC TCA AA

TaqMan primers used for qPCR	Target	Probe
Differentiation 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	*Hs 99999903_m1*

Embryoid body differentiation

Mechanically-detached iPSCs were plated in a Petri dish in floating condition. Nutristem-XF medium was then switched with DMEM F-12, 20% Knock-out serum replacement (Gibco), 0.1 mM β-mercaptoethanol, 1× NEAA, 50 U/ml Penicillin-Streptomicin, 2 mM L-glutamine in 3 days. EBs were grown in suspension for 14 days.

Immunofluorescence staining

Cells were fixed using 4% paraformaldehyde for 20 min at room temperature and the blocked in PBS containing 20% Normal Goat Serum and 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 O/N at 4 °C. After extensive washing, Alexa-Fluor-conjugated secondary antibodies were added for 1 h at room temperature. Cellular nuclei were counterstained with Hoechst. Microphotograps were taken using a Nikon C2 fluorescence microscope.

Teratoma formation

Approximately 3×10^6 Dispase-treated iPSC were resuspended in 100 μl of Matrigel. Cells were injected into immune-deficient mice, following ethical guidelines. Teratomas formed in 4–8 weeks. Upon removal, tumors were histologically analyzed to check for their in vivo differentiation capacity into derivatives of all three germ layers.

Karyotype analysis

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 cold fresh-made 3:1 ethanol:acetic acid solution. Karyotype analysis was carried out on GTG-banded metaphases. Fifteen metaphases were counted and three karyotypes analyzed (450–500 resolution). Only clonal aberrations were considered, 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.2018.02.016.

Acknowledgment

This work was supported by grants from the Italian Ministry of Health: Ricerca Corrente 2017 to JR.

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