

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.

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### Resource table.

Unique stem cell line identifier	CSSI003-A (2961)
Alternative name(s) of stem cell line	COL04 cIE2
Institution	Cellular Reprogramming Unit, IRCCS Casa Sollievo della Sofferenza – Viale dei Cappuccini, 71013 San Giovanni Rotondo, Foggia, Italy
Contact information of distributor	Jessica ROSATI <a href="mailto:j.rosati@css-mendel.it">j.rosati@css-mendel.it</a>
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

(continued)

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.

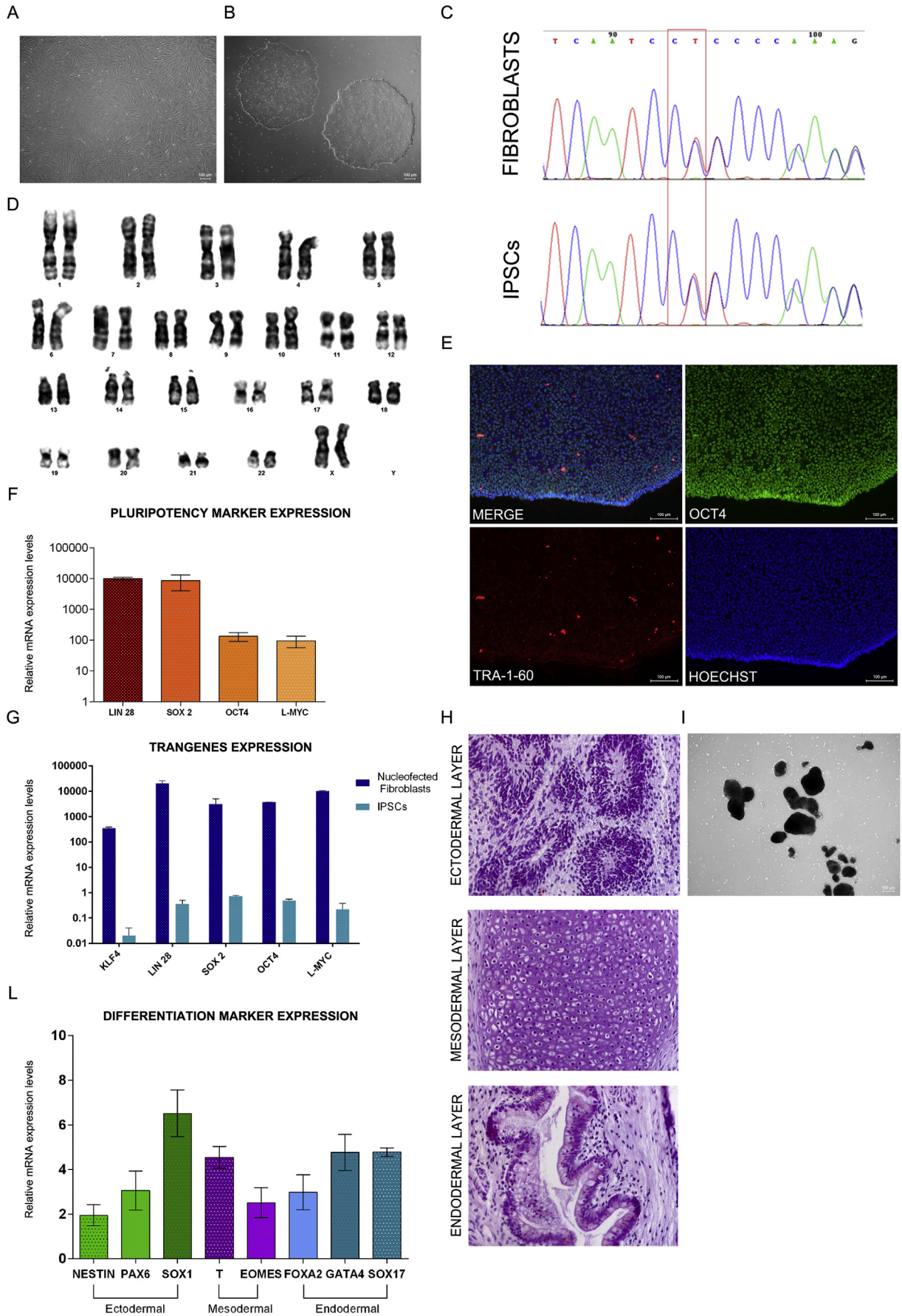
### Resource details

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

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**Table 1**  
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel B
Phenotype	Immunocytochemistry	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	46 XX,	Fig. 1 panel D
	(G-banding) and resolution	Resolution 450–500	
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.1194delC	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 embryoid 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 iPSC 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-Streptomycin and 1× Non-Essential Amino Acids (Sigma Aldrich) and cultured for 30 days to allow fibroblasts to grow.

Approximately  $1 \times 10^5$  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  $\beta$ -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  $\mu$ m. B. iPSC colonies morphology. Scale bar, 100  $\mu$ m. 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  $\mu$ m. 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  $\mu$ m. 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	$\beta$ -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	
	$\beta$ -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  $\beta$ -mercaptoethanol, 1 $\times$  NEAA, 50 U/ml Penicillin-Streptomycin, 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. Microphotographs were taken using a Nikon C2 fluorescence microscope.

### Teratoma formation

Approximately  $3 \times 10^6$  Dispase-treated iPSC were resuspended in 100  $\mu$ l 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  $\mu$ g/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<sup>+</sup>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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