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Lab Resource: Stem Cell Line

Generation of the Rubinstein-Taybi syndrome type 2 patient-derived induced pluripotent stem cell line (IAIi001-A) carrying the *EP300* exon 23 stop mutation c.3829A > T, p.(Lys1277*)

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A B S T R A C T

Rubinstein-Taybi syndrome (RSTS) is a neurodevelopmental disorder characterized by growth retardation, skeletal anomalies and intellectual disability, caused by heterozygous mutation in either the *CREBBP* (RSTS1) or *EP300* (RSTS2) genes. We generated an induced pluripotent stem cell line from an RSTS2 patient's blood mononuclear cells by Sendai virus non integrative reprogramming method. The iPSC line (IAIi001RSTS2-65-A) displayed iPSC morphology, expressed pluripotency markers, possessed trilineage differentiation potential and was stable by karyotyping. Mutation and western blot analyses demonstrated in IAIi001RSTS2-65-A the patient's specific non sense mutation in exon 23 c.3829A > T, p.(Lys 1277*) and showed reduced quantity of wild type p300 protein.

Resource table

Unique stem cell line identifier	IAIi001-A
Alternative name(s) of stem cell line	IAIi001RSTS2-65-A
Institution	Istituto Auxologico Italiano (IAI)-IRCCS, Milan, Italy
Contact information of distributor	Lidia Larizza, l.larizza@auxologico.it
Type of cell line	iPSC
Origin	Human
Additional origin info	Age: 25 years Sex: male Ethnicity: Caucasian
Cell source	Peripheral blood mononuclear cells (PBMCs)
Clonality	Clonal
Method of reprogramming	Sendai virus
Genetic modification	N/A
Type of modification	Spontaneous mutation
Associated disease	Rubinstein-Taybi syndrome type 2 (RSTS2)
Gene/locus	*EP300* gene c.3829A > T, p.(Lys1277*)
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	March 2017
	N/A

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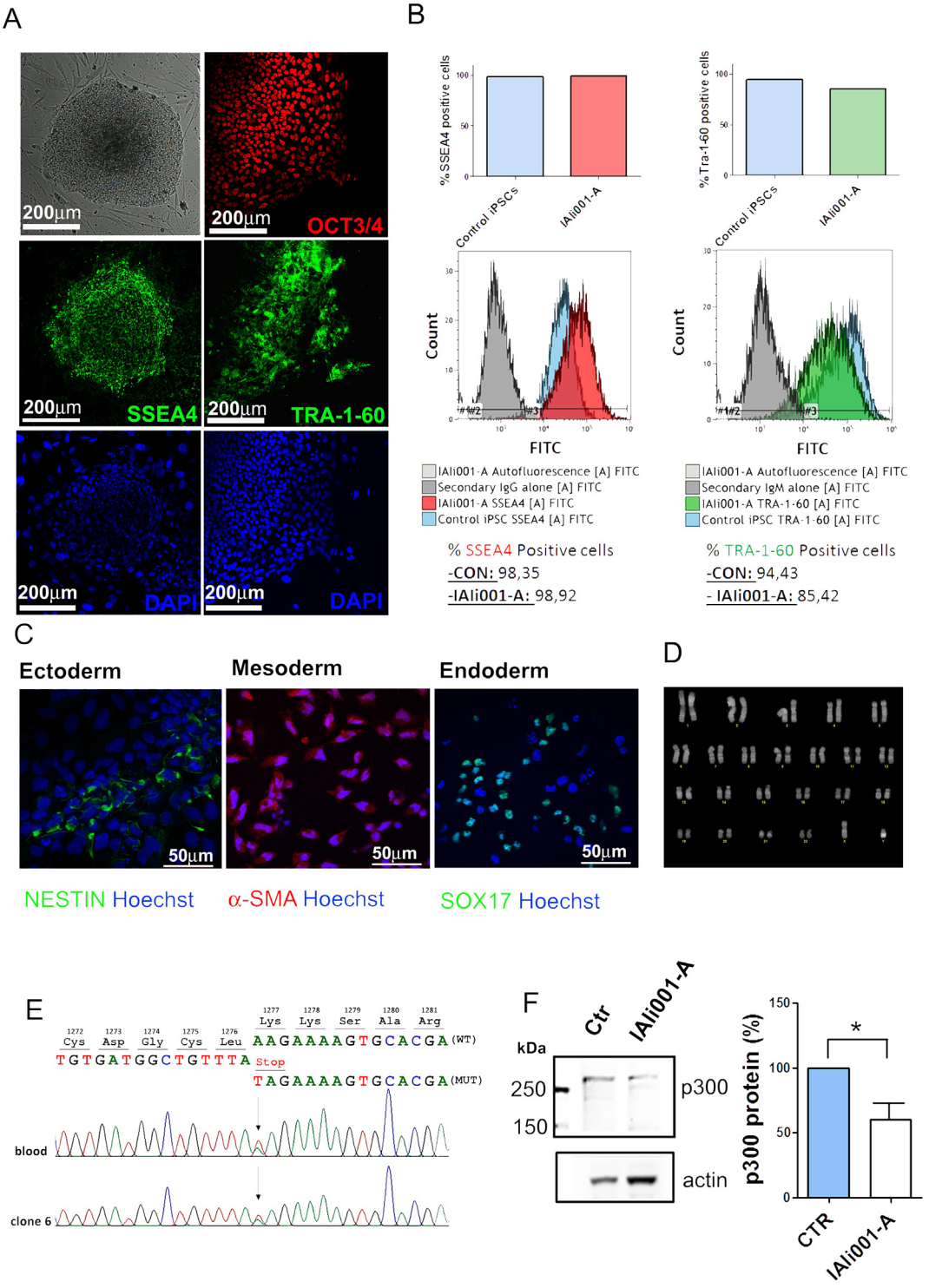

Fig. 1. Characterization of Rubinstein-Taybi syndrome type 2 induced pluripotent stem cell line (IAIi001-A).

Cell line repository/ bank	
Ethical approval	The study was approved by the ethical committee (CE) of Istituto Auxologico Italiano (IAI). CE code: 2015_12_15_02 Peripheral blood draw was obtained after informed consent was given.

Resource utility

This is the first iPSC line created for Rubinstein-Taybi syndrome. It provides a tool to disclose novel pathomechanisms downstream of mutation of *EP300* chromatin modifier gene and to identify biomarkers for epigenetic therapeutics of this rare neurodevelopmental disorder.

Resource details

Rubinstein-Taybi Syndrome (RSTS) is a rare multiple congenital

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1A
Phenotype	Immunocytochemistry	Positive for expression of pluripotency markers: SSEA4, OCT3/4, TRA-1-60	Fig. 1A
	Flow cytometry	Determined cell surface expression of SSEA4 (99%) and TRA-1-60 (85%)	Fig. 1B
Genotype	Karyotype (Q-banding) and resolution	46XY resolution: 400 band level	Fig. 1D
Identity	Microsatellite PCR (mPCR) OR STR analysis	Not performed	Available with Authors
	CNV analysis	Array-CGH detected CNVs (no.10) compared in IAIi001-A versus RSTS2–65A donor's PBMCs. 100% matching	
Mutation analysis	Sequencing	Confirmed heterozygous non sense mutation in exon 23 of *EP300* gene, c.3829A > T, p.(Lys1277*)	Fig. 1E
	Western blot	Confirmed reduced quantity (40%) of full length p300 protein	Fig. 1F
Microbiology and virology	Mycoplasma	Negative	Supplementary fig. S1
Differentiation potential	Directed differentiation	Determined the expression of markers for each of the three germ layers: NESTIN, ectoderm; αSMA, mesoderm; SOX17, endoderm	Fig. 1C

anomalies and intellectual disability syndrome characterized by growth retardation, skeletal anomalies and cognitive impairment, mainly caused by de novo heterozygous mutation in either *CREBBP* or *EP300* genes, encoding the homologous acetyltransferases and transcriptional coactivators CBP and p300 (Roelfsema and Peters, 2007). RSTS type 2 which clinical presentation is overall milder than RSTS1 (Fergelot et al., 2016) results from inactivating *EP300* mutations leading to p300 protein either reduced in quantity or defective in enzymatic function. Following institutional ethical committee approval and patient informed consent, peripheral blood was withdrawn from a 25-year-old male with RSTS2, who was previously clinically and molecularly described (Negri et al., 2016). Induced pluripotent stem cells (iPSCs) were generated from peripheral blood mononuclear cells (PBMCs) (Soares et al., 2015) using integration-free Sendai virus particles transducing target cells with replication-competent RNAs encoding the four reprogramming Yamanaka factors (Fusaki et al., 2009). iPSCs were grown on irradiated Mouse Embryonic Fibroblasts (MEFs) feeder layers. After 21 days from transduction iPSC colonies were manually selected and culture expanded. The iPSC line described here was named IAIi001RSTS2-65-A and was characterized by evaluating distinctive iPSC morphology and expression of the pluripotency markers by immunocytochemistry and FACS analyses (SSEA4, OCT3/4, TRA-1-60, Fig. 1A, 99% SSEA4$^+$cells, 85% TRA-1-60$^+$ cells, Fig. 1B) and potential to differentiate along ectodermal, mesodermal and endodermal lineages (NESTIN, αSMA and SOX17, Fig. 1C). Cytogenetic analysis, performed on > 30 mitoses, showed that the IAIi001RSTS2–65-A iPSC line was karyotypically normal at P6 (Fig. 1D). Sanger sequencing, performed on DNA extracted from IAIi001RSTS2–65-A revealed the *EP300* exon 23 non sense mutation c.3829A > T, (p.(Lys1277*)) (Fig. 1E). Western blot analysis showed a 40% reduced quantity of the full length p300 protein observed (band > 250 KDa) in control cells (Fig. 1F).

Materials and methods

Reprogramming of RSTS2 patient's erythroblasts to iPSCs

Following informed consent PBMCs were collected via gradient centrifugation from an RSTS2 patient with an *EP300* mutation c.3829A > T, (p.(Lys1277*)) and cultured for 9 days in enriched StemSpan™ Medium (Stemcell Technologies) at 37 °C in 5% CO_2. Reprogramming was performed by Sendai virus (Cytotune 2.0, LifeTech) (Soares et al., 2015) (Table 1).Transduced cells were plated on MEF feeders in HESC (human embryonic stem cell) medium (DMEM-F1220% KOSR, 1 mM L-glutamine, 1 × NEAA, 4 ng/ml FGF (all reagents from Life Technologies) and100 mM β-mercaptoethanol (Sigma))and fed every other day. Colonies were picked at day 20 and manually passaged weekly by cutting through the single colony in several places with a sterile syringe needle and then removing the colony by scraping it. Passage ratio is 1:5.iPSCs were harvested in 60% HESC medium, 30% FBS and 10% DMSO and stored in liquid nitrogen.

Pluripotency marker immunocytochemistry

IAIi001-RSTS2–65-A were fixed in 4% paraformaldehyde (20 min, 37 °C). Antibodies in gelatin dilution buffer (0.2% gelatin (for blocking), 0.3% Triton-×100 (for permeabilization), 20 mM Sodium Phosphate Buffer pH 7.4, 0.45 M NaCl, all by Sigma) were incubated at 4 °C overnight (primary) and 2 h at RT (secondary). Nuclei were counterstained with DAPI. Images were acquired with a Nikon Eclipse Ti microscope (Table 2).

Flow cytometry

iPSCs were dissociated in PBS/0.5 mM EDTA, fixed using BD Cytofix™ buffer (BD Biosciences) and stained with TRA-1-60 or SSEA4 antibody (both 1 h, 4 °C) followed by the specific fluorescently tagged secondary antibody (1 h 4 °C). Antibodies were diluted in 0,1% BSA, 0,5 mM EDTA in 1 × PBS solution. Cells were analyzed using a Gallios (Beckman Coulter) flow cytometer and Kaluza software. An iPSC line from a healthy donor was used as a characterization control.

In vitro trilineage differentiation potential assay

iPSCs were cultured on vitronectin-coated chamber slides and differentiated using the STEMdiff™ trilineage differentiation kit (Stemcell Technologies) according to the manufacturer's instructions (Table 2).

Karyotyping

Chromosomes, prepared at P6, after colcemid (10 μg/ml) overnight at 37 °C (5% CO2, 95% rH) were incubated in hypotonic solution (KCl 0.56%, 6 min, RT), washed 3 min with acetic acid 5% and fixed with methanol/acetic acid (3:1). Q-banded metaphases were photographed at 100× (Leica microscope and camera) and > 30 were karyotyped using CytoVision software (Leica).

Array-CGH analysis

High-resolution array comparative genomic hybridisation (array-CGH) was performed on genomic blood and iPSC DNA using the SurePrint G3 Human CGH Microarray Kit 4x180K in accordance with the manufacturer's instructions (Agilent Technologies). Data were then extracted and analyzed for copy number changes using Agilent CytoGenomics v.3.0.

Table 2
Antibodies used for immunocytochemistry/flow-citometry.

Antibodies used for immunocytochemistry/flow-citometry	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Rabbit anti-OCT3/4	1:200 (IF)	Santa Cruz Biotechnology Cat# sc-9081, RRID:AB_2167703
Pluripotency markers	Mouse anti-TRA-1-60	1:100 (IF)	Santa Cruz Biotechnology Cat# sc-21,705, RRID:AB_628385
Pluripotency markers	Mouse anti-SSEA4	1:100 (IF)	Thermo Fisher Scientific Cat# 14–8843-80, RRID:AB_657847
Pluripotency markers	Mouse anti-SSEA4	1:100 (FACS)	Abcam Cat# ab16287, RRID:AB_778073
Pluripotency markers	Mouse anti-TRA-1-60	1:100 (FACS)	Abcam Cat# ab16288, RRID: AB_ AB_778563
Differentiation markers (DM)	Mouse anti-NESTIN	1:100	Stemcell Technologies Cat# 60,091 CE, RRID:AB_2650581
Differentiation markers (DM)	Rabbit anti-SOX17	1:200	Cell Signaling Inc. Cat#81778, RRID:AB_2650582
Differentiation markers (DM)	Mouse anti-αSMA	1:200	Millipore Cat#CBL171, RRID:AB_2223166
Western immunoblot	Rabbit polyclonal anti-p300	1:300	Santa Cruz, N-15 Cat# sc 584, RRID: AB 2293429
Western immunoblot	Mouse monoclonal anti-actin antibody	1:2000	Sigma, AC40, Cat# A3853, RRID: AB 262137
Secondary antibodies	Anti-rabbit IG-HRP	1:2000	Millipore Cat# AP307P, RRID: AB 92641
Secondary antibodies	Anti-mouse IgG-HRP	1:2000	Millipore, Cat#AP124P, RRID: AB 90456
Secondary antibodies	F(ab')2-Goat anti- Rabbit IgG(H + L), Alexa®Fluor 555	1:300	Thermo Fisher Scientific Cat# A-21430, RRID:AB_2535851
Secondary antibodies	Anti-mouse IgG, Alexa®Fluor 488	1:500	Thermo Fisher Scientific Cat# A-11001, RRID:AB_2534069
Secondary antibodies	Anti-mouse IgG, Alexa®Fluor 488	1:400 (for SSEA4)	Thermo Fisher Scientific Cat# A11059, RRID:AB_2534106
Secondary antibodies	Anti-rabbit IgG, Alexa®Fluor 488	1:200 (for SOX17)	Thermo Fisher Scientific Cat# A11034, RRID:AB_2576217
Secondary antibodies	Anti-mouse IgM, Alexa®Fluor 488	1:200 (for TRA-1-60)	Thermo Fisher Scientific Cat# A21042, RRID:AB_AB_2535711
Secondary antibodies	Anti-mouse IgG2a Cross-adsorbed Secondary antibody, Alexa®Fluor 633	1:200 (for αSMA)	Thermo Fisher Scientific Cat# A-21136, RRID:AB_2535775

Primers	Target	Forward/reverse primer (5′-3′)
Targeted mutation analysis/sequencing	*EP300*_Ex23 c.3829A > T	F: ATGCCCTTCATGTTTCTTCATGTC R:TGCATTCTACAAATCCGTTCCTTG

EP300 mutation analysis by sanger sequencing

Genomic DNA was extracted using QIAmp DNA Mini kit (Qiagen). *EP300* exon 23 was amplified with GoTaq Flexi DNA polymerase (Promega) using exon flanking primers (95 °C-58 °C-72 °C, 35 cycles). Direct sequencing used the Big Dye Terminator v.1.1 Cycle Sequencing kit and ABI Prism 3130 Sequencer (Applied Biosystem). Electropherograms were analyzed with ChromasPro software 2.1.5 (Technelysium Pty Ltd). Sequence ENSG00000100393 was the *EP300* reference.

Western blot

Cells grown on vitronectin were detached with ReLeSR™ (Stemcell Technologies), pelleted and lysed in ice-cold 50 mM Tris-HCl (pH 7.4),150 mM NaCl, 0.5% Igepal, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.2 mM Na_3VO_4, protease and phosphatase inhibitors cocktail (Sigma/Aldrich). Nuclear proteins were released with DNase I (20 U) in 20 mM Tris-HCl (pH 7.4) 2.5 mM $MgCl_2$, 20 mM NaCl, and 1 mM PMSF (20 min, 4 °C) mixed with the soluble fraction in SDS-loading buffer and boiled (70 °C 10 min). Proteins (120 μg) were separated on NuPAGE 4–12% Bis-Tris Gel (Invitrogen), transferred to nitrocellulose and blocked with 5% BSA in PBS-0.2% Tween 20 (PBS-T). The membrane was incubated (1 h, RT) with antibodies to p300 and actin, and with HRP-labelled secondary antibodies (30 min, RT) (Table 2). Chemiluminescence signals were revealed with a Westar R imager (Hi-Tech Cyanagen). ImageJ was used for densitometric analyses.

Mycoplasma test

We ruled out the presence of Mycoplasma by using EZ-PCR Mycoplasma Test Kit (Biological Industries) according to the manufacturer's instructions. Positive Control was included in the kit.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.06.009.

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