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Lab Resource: Stem Cell Line

Generation and characterization of an induced pluripotent stem cell (iPSC) line from a patient with clozapine-resistant Schizophrenia

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ABSTRACT

Peripheral Blood Mononuclear Cells (PBMCs) were collected from a patient with clozapine-resistant (also known as "super-refractory") Schizophrenia. iPSCs were established with a non-integrating Sendai virus-based reprogramming system. A footprint-free hiPSC line was characterized to express the main endogenous pluripotency markers and to retain a normal karyotype. Cells showed pluripotency competency by giving rise to progeny of differentiated cells belonging to the three germ layers. This hiPSC line represents a valuable tool to obtain mature, pathology-relevant neuronal populations *in vitro* that are suitable to investigate the molecular background of the schizophrenic disorder and the resultant patients' response to treatments.

Resource table.

Name of stem cell line	SCZ#3-4 iPSC
Institution	University of Trento
Person who created resource	Silvio Scarone, Luciano Conti
Contact person and email	Silvio.Scarone@unimi.it; luciano.conti@unitn.it
Date archived/stock date	December 2015
Origin	Peripheral Blood Mononuclear Cells (PBMCs)
Type of resource	Induced pluripotent stem cells (iPSCs) derived from a schizophrenic (confirmed with SCID-I) Clozapine Non-Responder patient
Sub-type	Induced pluripotent stem cells (iPSCs)
Key transcription factors	hOCT4, hSOX2, hC-MYC, hKLF4 (CytoTune™-iPS 2.0 Sendai Reprogramming Kit - Thermo Fisher Scientific)
Authentication	Identity and purity of the cell lines was confirmed by SeV specific polymerase chain reaction (PCR), pluripotent proteins detection (Western Blot and immunocytochemistry), karyotyping, expression of specific markers of the three germ layers by means of in *in vitro* differentiation
Link to related literature	/
Information in public databases	/
Ethics	Patient informed consent obtained; Ethics Review Board-competent authority approval was obtained from the San Paolo Hospital Ethical Board

Resource details

Blood samples were collected by a 48-year old male patient with a diagnosis of disorganized and treatment-resistant Schizophrenia at the Department of Mental Health of the San Paolo Hospital, Milan (Italy). The diagnosis of Schizophrenia was confirmed by the assessment of two independent psychiatrists with the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I). A thorough review of the patient's history confirmed treatment resistance according to specific criteria (Caspi et al., 2004) but also resistance to clozapine, given lack of response to the compound and to available pharmacological augmentation strategies (Sommer et al., 2012).

To generate the SCZ#3-4 iPSC line the four Yamanaka reprogramming factors OCT4, SOX2, KLF4, and C-MYC (Takahashi et al., 2007) were delivered into PBMCs using the integration-free Sendai virus (Fusaki et al., 2009; Yang et al., 2008-2012) gene-delivery method (CytoTune-iPS 2.0 Sendai Reprogramming Kit; Thermo Fischer Scientific). iPSC-like colonies appeared after 10–12 days and were picked 6–7 days later (Fig. 1A). One of the clones gave rise to the stable expanding

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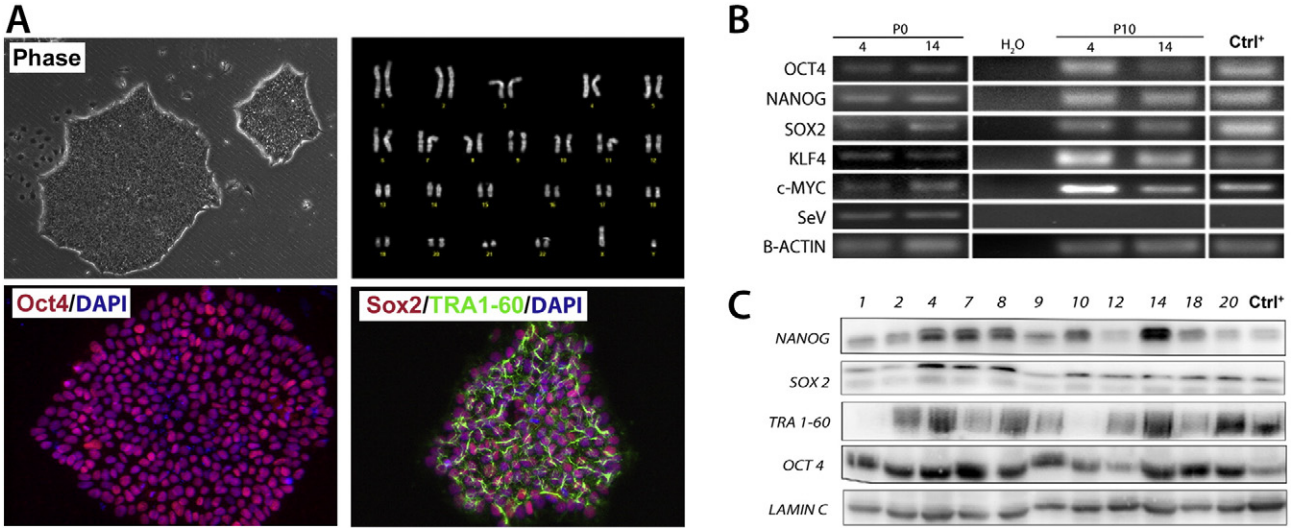

Fig. 1. Characterization of SCZ#3-4 iPSC line. **A**: Representative picture of a SCZ#3-4 hiPSC colony (5×) and its karyogram displaying a normal diploid 46, XY karyotype with no manifest cytogenetic abnormalities. Immunophenotypical characterization presenting the expression of the pluripotency markers OCT4, TRA-1-60, SOX2 (40×). **B**: RT-PCR showing the expression of the pluripotency-associated genes in passages 0 and 10 in SCZ#3-4 iPSCs cultures and in another hiPSC clone (#3-14) derived from the same patient. Lack of Sendai virus genome maintenance is presented in passage 10 cultures. A commercial hiPSC line was used as positive control for pluripotency-associated genes. **C**: Western Blot analysis showing protein expression levels of pluripotency-associated markers (NANOG, SOX2, TRA1-60, OCT4) in SCZ#3-4 iPSCs and in other clones derived from the same patient.

SCZ#3-4 iPSC line with a clear iPSC-like morphology (Fig. 1A) and uniform and specific OCT4, SOX2 and TRA1-60 immunoreactivity (Fig. 1A). Expression of pluripotency markers was also confirmed by PCR (Fig. 1B) and by Western Blot (Fig. 1C) analyses. SCZ#3-4 iPSC line displayed a normal diploid 46, XY karyotype, without appreciable abnormalities (Fig. 1B). The absence/presence of Sendai virus genome in cultures at passage 0 and passage 10 was analyzed by PCR and the loss of the viral genome was confirmed in passage 10 SCZ#3-4 iPSCs (Fig. 1B).

Pluripotent competence SCZ#3-4 iPSC line was assessed by Embryoid Body assay. Cells were cultured for 7 days in EB suspension and for additional 7 days in adhesion to promote the *in vitro* maturation towards the three germ layer derivatives (Carpenter et al., 2003). EBs cultures at 14 days displayed the presence of differentiated cells immunoreactive for ectodermal (β3-Tubulin), mesodermal (α-SMA) and endodermal (TROMA-1) markers (Fig. 2A). The differentiation competency of SCZ#3-4 iPSCs was comparable to that observed for a counterpart commercial hiPSC line, as shown by the similar expression levels of transcripts for FGF5 (ectoderm marker), Nestin (neuro-ectoderm marker), T-Brachyury (mesoderm marker), SOX-17 (endoderm marker) assessed by qRT-PCR (Fig. 2B).

Materials and methods

PBMCs collection and freezing

Peripheral Blood Mononuclear Cells (PBMCs) from patients were isolated in BD Vacutainer CPT Cell Preparation tubes with sodium citrate, after 30 min centrifugation (1800 ×*g* at room temperature). PBMCs were collected in PBS for a total volume of 35 ml and centrifuged

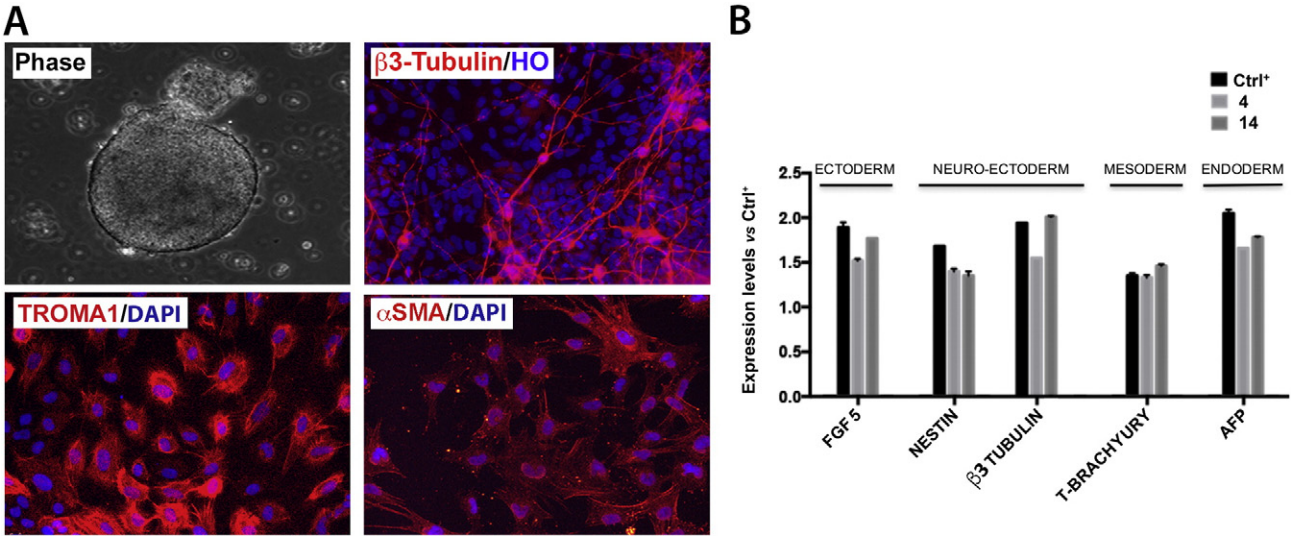

Fig. 2. *In vitro* differentiation SCZ#3-4 iPSC line. **A**: Embryoid Bodies formation assay after 4 days of suspension culture (5×). D14 cultures exhibit cells immunoreactive for ectodermal (β3Tubulin), mesodermal (α-SMA) and endodermal (TROMA-1) germ layer markers (20×). **B**: qRT-PCR showing an analogous expression levels of transcripts for the 3 germ-layers, FGF5 (ectoderm), Nestin and β3-Tubulin (neuro-ectoderm), T-Brachyury (mesoderm) and AFP (endoderm) between 14 days differentiated SCZ#3-4 and SCZ#3-14 iPSCs and differentiated reference commercial hiPSCs.

Table 1
List of primers sequences, amplicons size and number of PCR cycles.

Gene	Primer sequence		Amplicon size	Cycles (PCR)
BETA ACTIN	F: GACAGGATGCAGAAGGAGATTACTG	R: CTCAGGAGGAGCAATGATCTTGAT	72 bp	25
OCT4	F: GGAAGGAATTGGGAACACAAAGG	R: AACTTCACCTTCCCTCCAACCA	71 bp	30
SOX2	F: GCTACAGCATGATGCAGGACCA	R: TCTGCGAGCTGGTCATGGAGTT	135 bp	30
c-myc	F: CCTGGTGCTCCATGAGGAGAC	R: CAGACTCTGACCTTTTGCCAGG	128 bp	30
KLF4	F: CATCTCAAGGCACACCTGCGAA	R: TCGGTCGCATTTTTGGCACTGG	156 bp	30
NANOG	F: CCTGTGATTTGTGGGCCTG	R: GACAGTCTCCGTGTGAGGCAT	78 bp	30
SEV	F: GGATCACTAGGTGATATCGAGC	R: ACCAGACAAGAGTTTAAGAGATATGTATC	181 bp	35
Nestin	F: GGAGAAGGACCAAGAACTG	R: ACCTCCTCTGTGGCATTC	153 bp	qRT-PCR
T-Brachyury	F: CCTTCAGCAAAGTCAAGCTCACC	R: TGAACTGGGTCTCAGGGAAGCA	153 bp	qRT-PCR
FGF5	F: GGAATACGAGGAGTTTTCAGCAAC	R: CTCCCTGAACTTGCAGTCATCTG	99 bp	qRT-PCR
AFP	F: GCAGAGGAGATGTGCTGGATTG	R: CGTGGTCAGTTTGCAGCATTCTG	113 bp	qRT-PCR
β3-Tubulin	F: TCAGCGTCTACTACAACGAGGC	R: GCCTGAAGAGATGTCCAAAGGC	120 bp	qRT-PCR

at 300 ×*g* for 15 min RT and resuspended in fetal bovine serum (FBS) with 10% DMSO. 2×10^6 cells were aliquoted and frozen.

PBMCs thawing and reprogramming with Sendai virus particles

PBMCs were thawed at 37 °C and centrifuged at 200 ×*g* for 10 min in expansion medium (EM) made of StemPro-34 Serum Free Medium (SFM, Thermo Fisher Scientific) Basal Medium, StemPro-34 Nutrient Supplement, 200 mM GlutaMAX, 1% Penicillin/Streptomycin, 100 ng/ml Stem Cell Factor (SCF, Prepotech), 100 ng/ml FLT-3 (Thermo Fisher Scientific), 20 ng/ml Interleukin-6 (IL-6) (Thermo Fisher Scientific), 20 ng/ml Interleukin-3 (IL-3) (Thermo Fisher Scientific). The medium was replaced daily for the following 3 days.

In order to deliver reprogramming genes in PBMCs, viral particles provided with the CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific) were used following the manufacturer's protocol. 20 days post-transduction colonies with iPSCs morphology appeared and were picked, transferred onto a new well and cultured on Geltrex-coated plastic dish in E8 medium according to the manufacture's protocol.

In vitro differentiation

Embryoid Bodies (EB) formation assay was performed by gently resuspending iPSCs clumps in 100-mm non-tissue culture-treated dish in Essential 6 medium (E6 medium; Thermo Fisher Scientific). Medium was changed daily. At day 7, EBs were collected and plated on Geltrex-coated dishes in E6 medium to allow growth in adhesion for further 7 days. Medium was changed every other day.

RNA isolation, polymerase chain reaction (PCR) and quantitative-PCR (qPCR)

RNA was isolated with the TRIzol Reagent (Thermo Fisher Scientific) following the manufacture's protocol and reverse transcribed using iScript cDNA Synthesis Kit (BioRad). Transcripts of interest were amplified using EURO TAQ Thermostable DNA polymerase (EUROCLONE) and detection of genes of interest was confirmed with specific primes (Table 1). Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) was performed using the SsoAdvanced Universal SYBR Green Supermix Kit following the manufacturer's instructions. Beta-actin was used as housekeeping gene to normalize data. Amplification was performed on a CFX96 BioRad machine. Results were analyzed with BioRad CFX Manager dedicated software.

Immunofluorescence assay

Cells were fixed with PFA 4% for 15 min RT, permeabilized with Triton 0.5% for 15 minutes RT and blocked with blocking solution (10% FBS in PBS) for 1 h at RT. Cultures were then incubated with specific primary antibodies overnight at 4 °C (Table 2) and stained for 45 min at RT with secondary antibody and Hoechst 33258 1 μg/ml (Thermo Fischer Scientific). Images were detected with the microscope Leica DM IL Led Fluo with Leica DFC450 C camera (Leica Microsystem).

Western Blot assay

Cultures were lysed in SDS Sample Buffer (62.5 mM Tris-HCl ph 6.8; 2% SDS; 10% Glycerol; 50 mM DTT; Bromophenol Blue). Samples were

Table 2
List of the antibodies used in for immunocytochemistry (IC) and Western Blot (WB) assays, working dilution and species in which they are produced.

Antibody	Company	Dilution	Species
TRA1-60	Santa Cruz Biotech	1:1000 (WB) 1:200 (IC)	Mouse IgM
NANOG	Santa Cruz Biotech	1:1000	Mouse
SOX2	Millipore	1:2000 (WB) 1:300 (IC)	Rabbit
OCT4 (WB)	Santa Cruz Biotech	1:1000	Mouse
LAMIN A/C	Santa Cruz Biotech	1:1000	Rabbit
OCT4 (IC)	Santa Cruz Biotech	1:100	Rabbit
α-SMA	Sigma	1:100	Mouse
TROMA-1	Iowa DHB	1:100	Mouse
AFP	Abnova	1:50	Rabbit
β3-Tubulin	Promega	1:1000	Mouse
Anti-rabbit HRP	BioRad	1:3000	Goat
Anti-mouse HRP	BioRad	1:3000	Goat
Anti-mouse IgM FITC	Santa Cruz Biotech	1:200	Goat
Alexa Fluor IgG anti-rabbit 568	Life Technologies	1:300	Goat
Alexa Fluor IgG anti-rabbit 488	Life Technologies	1:300	Goat
Anti-mouse IgM FITC	Santa Cruz Biotech	1:200	Goat
Alexa Fluor IgG anti-mouse 568	Life Technologies	1:400	Donkey

boiled at 95 °C for 5 min and loaded in the 8% polyacrylamide gel and proteins blotted on a PVDF membrane by means of Trans Blot Turbo apparatus (BioRad). Primary antibodies (Table 2) were incubated overnight at 4 °C in agitation and secondary antibody for 45 min at RT. Signal was detected with the ECL Clarity system (BioRad) in dark chamber UVITECH Cambridge (Uvitech) and Uvitech software was used to acquire and analyze the data.

Karyotyping

Cell cultures were treated with colcemid (Gibco KaryoMAX Colcemid solution in PBS, Thermo Fischer Scientific) at a final concentration of 10 ng/ml for 16 h (overnight) at 37 °C and metaphases harvest was carried out according to standard protocols. Briefly, PBS washed cells were treated with hypotonic solution (0.075 M KCl for 15 min at RT) and fixed in acetic acid/methanol (1:3 v/v). Air-dried metaphase spreads slides were analyzed by QFQ banding following standard procedures. Microscope observation was performed using a Nikon Eclipse 90i (Nikon Instruments, Japan) equipped with the acquisition and analysis Genikon software (Nikon Instruments S.p.a. Italy).

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