Stem Cell Research 39 (2019) 101488

Contents lists available at ScienceDirect

Stem Cell Research

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

Lab resource: Stem Cell Line

Generation of an induced pluripotent stem cell line from a patient with autism spectrum disorder and *SCN2A* haploinsufficiency

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A B S T R A C T

Autism spectrum disorders (ASDs) are a group of diseases that affect social interaction, communication and behavior. Molecular mechanisms involved in the pathogenesis of ASDs are complex due to genetic heterogeneity. Recently, pathogenic variants of *SCN2A* have been strongly associated with ASDs. Here, we generated iPSCs from a patient with ASD and a heterozygous nonsense mutation in *SCN2A*, by reprogramming mesenchymal stromal cells with non-integrating vectors. The generated iPSC line expresses pluripotency markers, presents a normal karyotype and is able to differentiate into the three germ layers. This iPSC line is a useful tool for modeling ASD and drug screening studies.

Resource utility

Autism spectrum disorders (ASDs) are a group of diseases that affect social interaction, communication, and behavior (DSM-5, American Psychiatry Association, 2013). The incidence of ASDs is increasing worldwide and, in the USA, it is estimated to affect 1 in 59 children (CDC, 2018). There is significant heterogeneity both in the severity of the clinical presentation and in the molecular mechanisms that lead to the development of ASDs. In most cases, ASDs are associated with genetic factors, with dozens of genes described as susceptibility factors (Betancur, 2011). Pathogenic variants of *SCN2A* have shown a strong statistical association with ASDs (Ben-Shalom et al., 2017). *SCN2A* encodes the alpha subunit of the voltage-dependent sodium channel Nav1.2, which is highly expressed in pyramidal cells during development, playing roles in cortical organization, excitability and synaptogenesis (Sanders et al., 2018). Despite the statistical association between *SCN2A de novo* mutations and ASDs, further studies are needed in order to validate this correlation and increase pathophysiological knowledge. In this study, we generated an iPSC line from a patient diagnosed with ASD and carrier of a heterozygous point nonsense mutation in the *SCN2A* gene at position chr2: 165344558, with C > T substitution, which inserts a stop codon and interrupts the translation of the alpha subunit of $Na_v1.2$. This iPSC line is a useful tool for disease modeling studies to investigate the influence of *SCN2A* mutations in ASD, and to perform drug screening and gene editing.

Resource details

Bone marrow mesenchymal stromal cells (MSCs), previously obtained from the patient, were used for cell reprogramming. Written informed consent was obtained from the patient's parents. To generate iPSCs, MSCs were reprogrammed at P4, using Sendai Virus vectors (Cytotune 2.0, ThermoFisher Scientific). The resulting colonies were picked, expanded and analyzed for confirmation of the pluripotent state. The iPSCs showed the typical human embryonic stem cell (hESC)-like morphology (Fig. 1A, scale bar 100 μm), growing as compact round colonies. A high percentage of cells were positive for the pluripotency marker TRA-1-60, as shown by flow cytometry analysis (Fig. 1B). This finding was complemented by immunostaining for TRA-1-60, OCT3/4 and SOX2 (Fig. 1C, scale bar 25 μm). The expression of endogenous pluripotency genes was detected by RT-PCR (Fig. 1D). The elimination of Sendai vectors' transgenes was confirmed by RT-PCR for SeV at P15 (Fig. 1E). G-band karyotype analysis was performed for evaluation of chromosomal stability in iPSCs at P7 (Fig. 1F) and showed no numerical or structural clonal alterations. The iPSC line was also re-evaluated by Sanger sequencing at the mutation locus, which confirmed the presence

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https://doi.org/10.1016/j.scr.2019.101488
Received 10 May 2019; Received in revised form 3 June 2019
Available online 25 June 2019

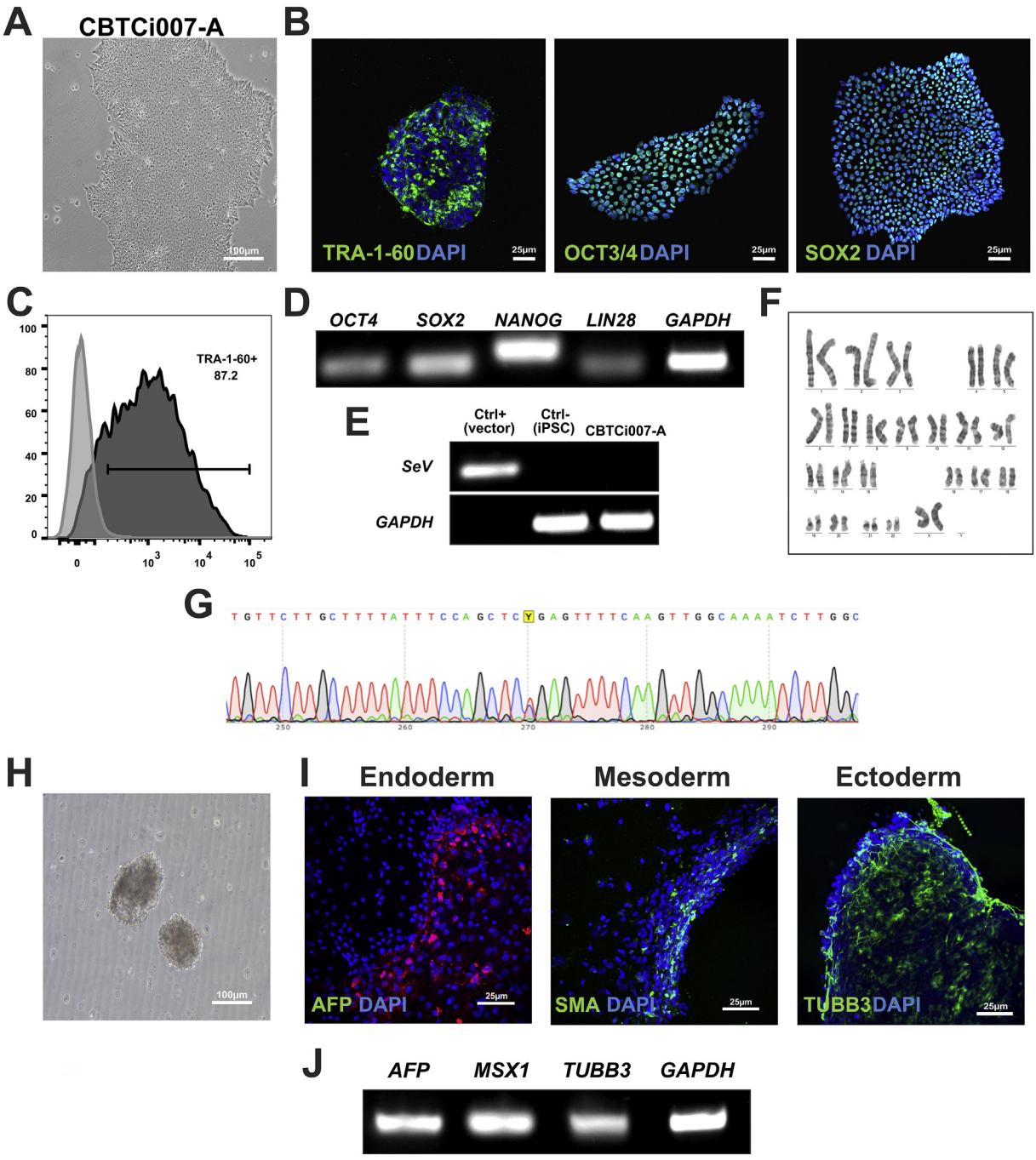

Fig. 1. Generation and characterization of iPSC line from a patient with ASD and SCN2A haploinsufficiency.

of a heterozygous point mutation in the *SCN2A* gene at position chr2: 165344558, with C > T substitution, that inserts a stop codon (Fig. 1G). The iPSC line obtained was able to generate derivatives of the three-germ layers in an embryoid body (EB) formation assay. EBs presented spherical morphologic, growing in suspension (Fig. 1H, scale bar 100 µm) and were positive for differentiation markers of ectoderm (TUBB3), mesoderm (smooth muscle actin, alpha-SMA) and endoderm (alpha fetoprotein, AFP) (Fig. 1I, scale bar 25 µm). The expression of markers for three germ layer differentiation was also confirmed by RT-PCR for *AFP* (endoderm), *MSX1* (mesoderm), and *TUBB3* (ectoderm) (Fig. 1J). Contamination with *Mycoplasma* sp. was excluded by PCR analysis (Supplementary Fig. 1). Finally, we also confirmed the genetic identity of the cell line to the parental MSCs through STR analysis (available with the authors).

Materials and methods

Cell reprogramming

MSCs were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and maintained at an incubator at 37 °C in a humidified atmosphere containing 5% CO_2. MSCs were dissociated with trypsin-EDTA (ThermoFisher), resuspended in 10% FBS-DMEM, centrifuged at 1500 RPM for 5 min, the supernatant was discarded, and the cells were resuspended in 10% FBS-DMEM. Then, 2×10^5 cells were plated in one well of a 6-well plate and cultured overnight. On the following day (D0), the cells were infected with Sendai Virus (Cytotune 2.0 Kit, ThermoFisher Scientific) using the multiplicities of infection (MOIs): 5:5:5 of the hCOS viruses, cMYC and KLF4 respectively. On D1, the

medium was exchanged to fresh 10% FBS-DMEM medium. From D2-D7, 0.5 mM sodium butyrate (NaBut) was added to the medium, and complete medium changes were performed every other day. On D8, the cells were trypsinized and plated into Matrigel-coated wells (Corning) of a 6-well plate, splitted in the ratio 1:2, with mTeSR1 (StemCell Technologies), which was supplemented, between D8-D14, with the following small molecules: 0.5 mM NaBut, 0.5 μM Thiazovivin, 0.5 μM PD0325901 and 2 μM SB431542. The medium was changed daily, and, at D15, it was possible to observe the appearance of colonies. The cells were then dissociated with Accutase (StemCell Technologies), and re-plated in 24 Matrigel-coated wells of a 24-wells plate. The Rho kinase inhibitor Y-27632 at 10 μM was added to the medium in the first 24 h after single-cell passaging. The cells were maintained in mTeSR1 with daily medium exchanges and at least one colony was observed in each well in the following days. Colonies were manually picked based on morphology, plated into plates coated with Matrigel hESC-qualified matrix (Corning), and cultured in mTeSR1 medium (StemCell Technologies), with daily media exchanges. The iPSCs were routinely passaged using 0.5 mM EDTA solution, once a week, with 1:10 split ratio, or twice a week with 1:6 split ratio, being cryopreserved in liquid nitrogen in KnockOut Serum Replacement (ThermoFisher Scientific) containing 10% DMSO.

In vitro differentiation by embryoid body (EB) formation assay

iPSCs were dissociated with Accutase and resuspended in 1 mL of EB medium, composed of DMEM F12 supplemented with 20% KSR, 1% Non-Essential Amino Acids, 1× Glutamax, 1% Penicillin/Streptomycin (all from ThermoFisher Scientific) and 10 μM Y-27632 (StemCell Technologies). Next, 1 mL of a 1×10^6 cells/mL cell suspension was transferred to an Aggrewell plate (StemCell Technologies) for EB formation, according to manufacturer's instructions. Half of the media was replaced after two days. After one week, the aggregates were replated into a non-adherent 60 mm plate, where the cells were maintained for seven days. Then, the EBs were plated into 24 well-plates coated with Matrigel (Corning) for adhesion. The cells were maintained with EB medium for one week and then were fixed with 4% PFA for immunofluorescence analysis or incubated with Trizol (ThermoFisher Scientific) for RNA extraction and RT-PCR assays (Table 1).

Immunofluorescence (IF) analysis

iPSC cells were plated on coverslips coated with Matrigel (Corning). Later, the cells were fixed with 4% PFA for 15 min and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 15 min at RT. Non-specific binding was blocked with 5% BSA in PBS for 30 min. Primary antibodies for the pluripotency markers OCT-4, SOX2 and TRA-1-60, diluted in 0.1% BSA in PBS, were added and the cells were incubated overnight at 4 °C. On the following day, the cells were incubated with the secondary antibodies for 40 min at RT. Nuclei were stained with DAPI (Vector Labs). Staining for three germ layer markers on EBs was performed with anti-AFP, anti-SMA, and anti-Nestin as primary antibodies. All dilutions and antibodies manufacturers are described in Table 2. Slides were observed and images were acquired using A1 confocal microscope (Nikon).

Flow cytometry

iPSCs were dissociated using Accutase (ThermoFisher Scientific), and incubated with the antibody TRA-1-60-Alexa Fluor 647 or Mouse IgM-Alexa Fluor 647 Isotype Control (BD Biosciences) diluted in PBS for 15 min at RT. The cells were washed and resuspended in PBS + Hoechst 33342 (2 μg/mL) for live cell data acquisition using LSR Fortessa SORP flow cytometer (BD Biosciences). Data analysis was performed using FlowJo v.X (Tristar).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from iPSC cells using TRIZOL (ThermoFisher Scientific) and 1 μg of RNA was used for cDNA synthesis using the Multiscribe™ kit according to the manufacturer's instructions. The cDNA was treated with DNAse (Ambion) and then used to perform a PCR for LIN28, SOX2, NANOG, OCT3/4, AFP, MSX1, TUBB3 and SeV and the housekeeping gene GAPDH, using primers described in Table 2. PCR products were visualized by electrophoresis on agarose gel 1%.

PCR condition for LIN28: 94 °C 2 min, 94 °C 30 s, 65 °C 30 s, 72 °C 1 min (30×), 72 °C 10 min.

PCR condition for SOX2: 94 °C 2 min, 94 °C 30 s, 62 °C 30 s, 72 °C 1 min (30×), 72 °C 10 min.

PCR condition for NANOG: 94 °C 2 min, 94 °C 30 s, 52 °C 30 s, 72 °C 1 min (30×), 72 °C 10 min.

PCR condition for OCT3/4: 94 °C 2 min, 94 °C 30 s, 58 °C 30 s, 72 °C 1 min (30×), 72 °C 10 min.

PCR condition for AFP: 94 °C 2 min, 94 °C 30 s, 62 °C 30 s, 72 °C 1 min (35×), 72 °C 10 min.

PCR condition for MSX1: 94 °C 2 min, 94 °C 30 s, 62 °C 30 s, 72 °C 1 min (35×), 72 °C 10 min.

PCR condition for TUBB3: 94 °C 2 min, 94 °C 30 s, 62 °C 30 s, 72 °C

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Phase-contrast images of iPSC colonies	Normal	Fig. 1, panel A
Phenotype	Qualitative analysis (immunofluorescence and PCR)	The iPSC cell lines show expression of the pluripotency markers: OCT3/4, SOX2, TRA-1-60 (IF) and *OCT4*, *SOX2*, *NANOG*, *LIN28*, (RT-PCR)	Fig. 1 panel C (IF); Fig. 1 panel D (PCR)
	Quantitative analysis (flow cytometry)	Tra-1-60–87.2%	Fig. 1 panel B
Genotype	Karyotype (G-banding) and resolution	46XX Resolution 450–500 band	Fig. 1 panel F
Identity	Microsatellite PCR (mPCR)	N/A	N/A
	STR analysis	22 loci analyzed by STR matched between parental cells and respective iPSC lines.	Data available with the authors.
Mutation analysis	Sequencing	Heterozygous *SCN2A*/chr2: 165344558, with C > T substitution, (c.2566C > T, p.Arg856Ter*	Fig. 1, panel G
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma by PCR	Negative	Supplementary Fig. 1
Differentiation potential	Embryoid body formation	*In vitro* differentiation showing expression of *AFP*, *MSX1*, *TUBB3* (RT-PCR) and AFP, SMA and Nestin (IF)	Fig. 1 panel J (PCR); Fig. 1 panel I (IF)
Donor screening (OPTIONAL)	N/A	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	TRA-1-60-Alexa Fluor 647 (mouse)	1:500	BD Biosciences Cat# 560122, RRID: AB_1645448
	Mouse IgM – Alexa Fluor 647 Isotype Control (mouse)	1:500	BD Biosciences Cat# 560806, RRID: AB_2034030
	TRA-1-60 (mouse)	1:500	Abcam Cat# ab16288, RRID: AB_778563
	Oct4 (rabbit)	1:500	Abcam Cat# ab181557, RRID: AB_2687916
	Sox2 (goat)	1:100	Santa Cruz Biotechnology Cat# sc-17320, RRID: AB_2286684
Differentiation Markers	AFP (rabbit)	1:300	Innovative Research Cat# 18-0055, RRID: AB_138884 Dako Cat# M0851, RRID: AB_2223500
	SMA (mouse)	1:300	Millipore Cat# MAB5326, RRID: AB_2251134
	TUBB3 (mouse)	1:300	Biolegend Cat#801201, B209227
Secondary antibodies	Goat anti-Mouse IgM Alexa Fluor 488	1:1000	Thermo Fisher Scientific Cat# A-21042, RRID: AB_2535711
	Goat anti-Mouse IgG Alexa Fluor 488	1:1000	Thermo Fisher Scientific Cat# A-11017, RRID: AB_2534084
	Goat anti-Rabbit IgG Alexa Fluor 488	1:1000	Thermo Fisher Scientific Cat# A-11008, RRID: AB_143165
	Donkey anti-Rabbit IgG Alexa Fluor 568	1:1000	Thermo Fisher Scientific Cat# A10042, RRID: AB_2534017
	Rabbit anti-Goat IgG Alexa Fluor 568	1:1000	Thermo Fisher Scientific Cat# A-11079, RRID: AB_2534123

Primers		
	Target	Forward/Reverse primer (5′-3′)
Viral Vector (PCR)	*SeV* (181 bp)	GGA TCA CTA GGT GAT ATC GAG C ACC AGA CAA GAG TTT AAG AGA TAT GTA TC
Pluripotency Markers (RT-PCR)	*NANOG* (78 bp)	CCT GTG ATT TGT GGG CCT G GAC AGT CTC CGT GTG AGG CAT
	LIN28 (129 bp)	AGC CAT ATG GTA GCC TCA TGT CCG C TCA ATT CTG TGC CTC CGG GAG CAG GGT AGG
	SOX2 (80 bp)	TTC ACA TGT CCC AGC ACT ACC AGA TCA CAT GTG TGA GAG GGG CAG TGT GC
	POU5F1 (143 bp)	CCC CAG GGC CCC ATT TTG GTA CC ACC TCA GTT TGA ATG CAT GGG AGA GC
House-Keeping Genes (RT-PCR)	*GAPDH* (452 bp)	ACC ACA GTC CAT GCC ATC AC TCC ACC ACC CTG TTG CTG TA
Differentiation Markers (RT-PCR)	*TUBB3* (148 bp)	GCT CAG GGG CCT TTG GAC ATC TCT T TTT TCA CAC TCC TTC CGC ACC ACA TC
	MSX1 (307 bp)	CGA GAG GAC CCC GTG GAT GCA GAG GGC GGC CAT CTT CAG CTT CTC CAG
	AFP (281 bp)	GAA TGC TGC AAA CTG ACC ACG CTG GAA C TGG CAT TCA AGA GGG TTT TCA GTC TGG A
Targeted mutation analysis/sequencing	*SCN2A* (1496 bp)	CTC CTT TGG GTC ACT TTG ATG C AGG GTT TGT GTG GAT TAC AAT GG
Mycoplasma by PCR	*Mycoplasma sp.* (1000 bp)	ATT AGA TAC CCT GGT AGT CCA CG GAC TAC CAG GGT ATC TAA TCC TG

1 min (35×), 72 °C 10 min.

PCR condition for SeV: 94 °C 2 min, 94 °C 30 s, 55 °C 30 s, 72 °C 1 min (30×), 72 °C 10 min.

Short tandem repeat (STR) analysis

STR analyses were performed by pairing the parental cell source, patient-derived MSCs, and the iPSC line. Briefly, genomic DNAs were isolated using Puregene® Core Kit A, amplified by PowerPlex Fusion System (Promega) and then analyzed with ABI3500 (Applied Biosystems) genetic analyzer using the software program GeneMapper v.5.0 (Applied Biosystems).

Karyotype analysis

iPSCs were analyzed for chromosomal alterations by G-band karyotyping at P7. The cells were incubated with colcemid (0,1 μg/mL) for 1 h at 37 °C (5% CO2, 95% rH). The cells were then incubated in hypotonic solution (KCl 0.75%, 15 min, 90 °C), washed 3 min with acetic acid 5% and fixed with methanol/acetic acid (3:1). G-banded metaphase images were acquired with a 100× objective (Olympus BX61 microscope and ProgRes MFcool camera). Analysis was conducted in 20 metaphases using Lucia Karyo software (Lucia Cytogenetics) with a 400–450 band resolution.

Mycoplasma testing

Absence of *Mycoplasma* contamination was confirmed by PCR. To prepare the samples, 1 mL of cell culture supernatant was collected, centrifuged at 10000 RPM for 15 min, the supernatant was discarded, and 50 μL of 10 mM Tris/HCl was added, followed by vortexing for 10 s and incubation at 95 °C for 10 min. The samples were maintained at −20 °C until used to perform the PCR using primers described in Table 2. PCR products were visualized by electrophoresis on agarose gel 1%. PCR conditions for *Mycoplasma* detection: 95 °C 3 min, 96 °C 20 s, 55 °C 30 s, 72 °C 30 s (45×).

Key resources table

Unique stem cell line identifier	CBTCi007-A
Alternative name(s) of stem cell line	iM5
Institution	CBTC
Contact information of distributor	Bruno Solano - brunosolanosouza@gmail.com
Type of cell line	iPSC
Origin	Human

Additional origin info	Applicable for human iPSC Age: 4 Sex: female
Cell source	Bone marrow mesenchymal stromal cells
Clonality	Clonal
Method of reprogramming	Sendai virus (Cytotune 2.0)
Genetic Modification	N/A
Type of Modification	N/A
Associated disease	Autism
Gene/locus	*SCN2A*/chr2: 165344558, with C > T substitution, (c.2566C > T, p.Arg856Ter * CCDS33313.1)
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	N/A
Cell line repository/bank	N/A
Ethical approval	CAAE 19883113.0.0000.0048

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101488.

Acknowledgements

This research was funded by FAPESB and CAPES.

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