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Lab Resource: Stem Cell Line

Generation of induced pluripotent stem cells (iPSC) from an atrial fibrillation patient carrying a PITX2 p.M200V mutation

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ABSTRACT

Atrial fibrillation (AF) is the most common sustained arrhythmia associated with several cardiac risk factors, but increasing evidences indicated a genetic component. Indeed, genetic variations of the specific PITX2 gene have been identified in patients with early-onset AF. To investigate the molecular mechanisms underlying AF, we reprogrammed to pluripotency polymorphonucleated leukocytes isolated from the blood of a patient carrying a PITX2 p.M200V mutation, using a commercially available non-integrating expression system. The generated iPSCs expressed pluripotency markers and differentiated toward cells belonging to the three embryonic germ layers. Moreover, the cells showed a normal karyotype and retained the PITX2 p.M200V mutation.

Resource Table

Unique stem cell line identifier	UNIBSi003-A
Alternative name(s) of stem cell line	MI37
Institution	Department of Molecular and Translational Medicine, University of Brescia, 25123 Brescia, Italy
Contact information of distributor	Patrizia Dell'Era; patrizia.dellera@unibs.it
Type of cell line	iPSC
Origin	Human
Additional origin info	Age:24 Sex: male Ethnicity: Caucasian
Cell source	Blood
Method of reprogramming	CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific). The episomal reprogramming vectors include the four Yamanaka factors, Oct, Sox2, Klf4, and c-Myc
Genetic modification	NO
Type of modification	N/A
Associated disease	Paroxysmal atrial fibrillation
Gene/locus	PITX2 (NM_001204397.1) gene p.M200V (c.598 A>G) mutation in exon 7. gDNA Chr4(GRCh38): g.110618481T>C
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	August 2015
Cell line repository/bank	NO
Ethical approval	Written informed consent was obtained from all study participants. The study was approved by the scientific ethics committee for the Capital Region of Denmark (protocol number H-1-2011-044)

Resource utility

We generated a human cellular atrial fibrillation model starting from a patient carrying PITX2 p.M200V mutation, already described to segregate with lone AF (Christophersen et al., 2013). We believe that the analysis of iPSC-derived mutated cardiomyocytes will contribute to elucidate the molecular mechanisms underlying AF.

Resource details

UNIBSi003-A cell line was generated from patient's isolated polymorphonuclear leukocytes under feeder-free culture conditions.

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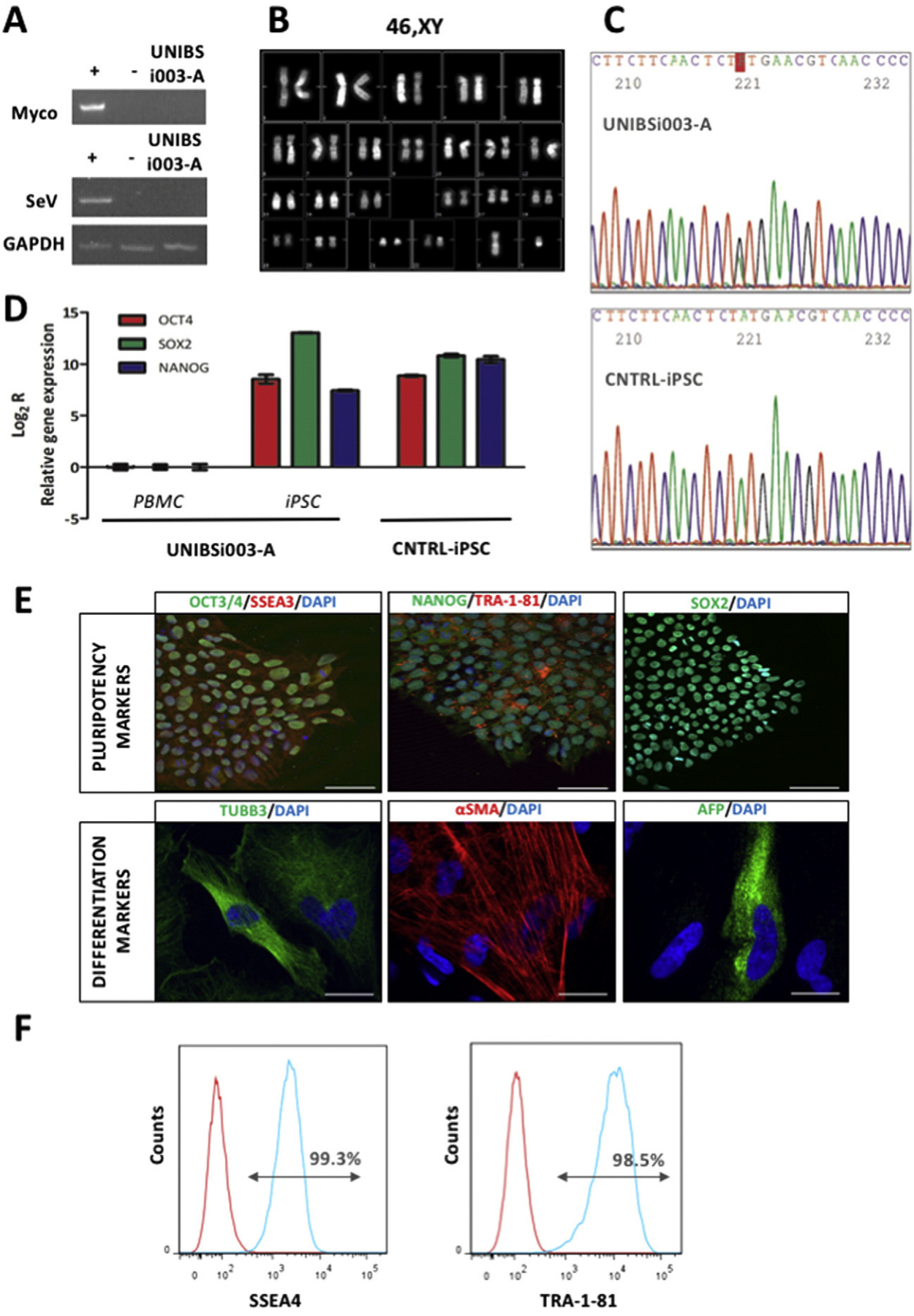

Fig. 1. Molecular and genetical analysis of UNIBSi003-A iPSC line.

To obtain iPSC cells we infected blood cells using the CytoTune 2.0 iPS Sendai Reprogramming Kit (Thermo Fisher Scientific) which employs the non-integrating Sendai virus (SeV) to deliver Yamanaka's factors OCT4, SOX2, KLF4, and cMYC. Once the cell line was stabilized we evaluate the presence of contaminating mycoplasma by PCR analysis as well as the loss of the SeV used for the reprogramming procedure by reverse transcriptase PCR. Results are illustrated in Fig. 1A and indicate that UNIBSi003-A cell line is both mycoplasma- and SeV-free. The presence of numerical or structural chromosome abnormalities was evaluated using standard QFQ-banding that revealed a normal 46, XY karyotype of the cell line (Fig. 1B). Moreover, the persistence of the patient's DNA mutation was confirmed by iPSC Sanger sequencing. As shown in Fig. 1C, UNIBSi003-A iPSCs carry the heterozygosity in PITX2 gene that leads to the p.M200V mutation.

Next, the expression of the endogenous pluripotent transcription factors was evaluated by quantitative PCR, using the primers reported in Table 2, while the presence of the related proteins, as well as the presence of additional stem cell markers, was evaluated by immunofluorescence staining of iPSC colonies using the antibodies listed in Table 2. At variance with PBMC, UNIBSi003-A iPSCs express high levels of endogenous OCT4, SOX2, and NANOG, fully comparable to those of a control iPSC line (Fig. 1D). Moreover, the resulting proteins are correctly shown in the nuclear compartment of the cells, while additional SSEA3 and Tra-1-81 markers are properly present on cell surface (Fig. 1E). Flow cytometry using SSEA4 and Tra-1-81 antibodies revealed a pluripotent population higher than 98.5% (Fig. 1F).

Following the verification of pluripotency marker expression, we investigated the UNIBSi003-A iPSC spontaneous differentiation capacity in the three-dimensional structures called embryoid bodies (EBs). EBs were left in suspension for seven days and then allowed to adhere to a standard tissue culture surface. After two weeks, cells were fixed and immunofluorescence was performed. The in vitro EB formation assay confirmed the spontaneous differentiation capacity toward the three germ layers of the UNIBSi003-A iPSC as demonstrated by the expression of ectodermal (TUBB3), mesodermal (alpha-smooth muscle actin, α-SMA) and endodermal (alpha-fetoprotein, AFP) markers (Fig. 1E).

In conclusion, we generated an iPSC line carrying the PITX2 gene p.M200V variant that has been previously described to co-segregate with AF. In Table 1, STR analysis that uniquely identify UNIBSi003-A iPSCs has been reported. Potentially, the study of the molecular basis of lone AF will strongly benefit by the analysis of the cardiomyocytes derived from UNIBSi003-A iPSCs, where the mutation is inserted in the correct pathological genetic background of the patient.

Materials and methods

Reprogramming of peripheral blood mononuclear cells (PBMC)

Peripheral blood was collected from a clinically diagnosed 38-years-old male patient suffering from paroxismal AF harboring the PITX2 p.M200V mutation in exon 7. PBMC were isolated by Ficoll-Paque® PLUS density gradient centrifugation using SepMate™-15 - specialized PBMC Isolation Tube (Stem Cell Technologies) and maintained in StemPro®-34 SFM Medium (Thermo Fisher Scientific) supplemented with StemSpam CC100 cytokines (Stem Cell Technologies) for 4 days.

Then, PBMCs were transduced using the CytoTune 2.0 iPS Sendai Reprogramming Kit (Thermo Fisher Scientific) following manufacturer's instructions. hiPSC colonies were manually picked between day 17 and day 28 post infection, and expanded for further characterization.

iPSC karyotyping

Cells undergoing active cell division were blocked at metaphase by adding 10 μg/mL of colcemid (Karyo Max, Gibco Co. BRL) to culture medium for 3 h at 37 °C. Then, cells were trypsinized, swollen by exposure to hypotonic 75 mM KCL solution, fixed with methanol/glacial acetic acid (3:1), and dropped onto glass slides. Cytogenetic analysis was performed using QFQ-banding at 400–450 bands resolution according to the International System for Human Cytogenetic Nomenclature (ISCN 2016). A minimum of 20 metaphase spreads and 3 karyograms were analyzed.

iPSC genotyping

Genomic DNA from patient-derived iPSC was extracted using DNeasy blood and tissue kit (Qiagen) and the analysis of the PITX2 (NM_001204397.1) mutation was performed using a primer pair, described in Table 2, spanning the p.M200V (c.598 A>G) mutation in exon 7 of the PITX2 gene.

RNA extraction and RT-PCR analysis

Total RNA was extracted using Quick-RNA MiniPrep (Zymo Research), and quantified. Reverse transcriptase-PCR of 1 μg of total RNA was carried out using with iScript cDNA Synthesis Kit (BIO-RAD), followed by specific PCR amplification. Sequences of individual primer pairs are detailed in Table 2. PCR products were visualized by agarose/ethidium bromide gel electrophoresis.

Table 1
Characterization and validation of UNIBSi003-A iPSC line.

Classification	Test	Result	Data
Morphology	Photography	Visual record of the line: normal	Not shown but available with author
Phenotype	Immunocytochemistry	Expression of pluripotency markers OCT4, SOX2, NANOG, SSEA3, and Tra-1-81	Fig. 1 panel E
		Expression of pluripotency markers SSEA4 and Tra-1-81	Fig. 1 panel F
	Flow citometry	Expression of pluripotency markers SSEA4: 99.3% and Tra-1-81: 98.5%.	Fig. 1 panel F
Genotype	Karyotype (QFQ-banding) and resolution	46XY, at 400–450 band resolution	Fig. 1 panel B
Identity	STR analysis	22 sites tested	Supplementary file
Mutation analysis	Sequencing	Heterozygous	Fig. 1 panel C
Microbiology and virology	Mycoplasma	Absence of mycoplasma detected by PCR analysis	Fig. 1 panel A
Differentiation potential	Embryoid body formation	Immunocytochemical expression of alpha fetoprotein (endodermal germ layer), beta III Tubulin (ectodermal germ layer), and alpha Smooth Muscle Actin (mesodermal germ layer)	Fig. 1 panel E

Table 2
Reagents details.

Antibodies used for immunocytochemistry and flow cytometry			
	Antibody	Dilution	Company Cat# and RRID
Pluripotency marker	Mouse anti-OCT3/4	1:60	Santa Cruz, Cat#sc5279, RRID: AB_628051
Pluripotency marker	Mouse anti-SOX2	1:200	R&D, Cat#MAB2018
Pluripotency marker	Goat anti-NANOG	1:200	Everest Biotech, Cat#EB06860 RRID: AB_2150379
Pluripotency marker	Mouse anti-Tra-1-81	1:200	Millipore, Cat#MAB4381 RRID: AB_177638
Pluripotency marker	Rat anti-SSEA3	1:100	DSHB, Cat#MC-631 RRID: AB_528476
Pluripotency marker	Mouse anti-SSEA4	1:200	R&D, Cat#FAB1435P RRID: AB_357038
Differentiation markers	Rabbit anti-AFP	1:400	DAKO, Cat#A0008, RRID: AB_2650473
Differentiation markers	Mouse anti-βIIITub	1:500	Sigma-Aldrich, Cat#T8660, RRID: AB_477590
Differentiation markers	Mouse anti-αSMA	1:300	Thermo Fisher Scientific/Lab Vision, Cat# MS-113-P1, RRID: AB_64002
Secondary antibodies	Goat anti-rat IgM 594	1:600	Invitrogen, Cat#A21213, RRID: AB_11180463
Secondary antibodies	Goat anti-mouse IgM 594	1:600	Invitrogen, Cat#A21044 RRID: AB_2535713
Secondary antibodies	Donkey anti-mouse IgG 488	1:600	Invitrogen, Cat#A21202 RRID: AB_2535788
Secondary antibodies	Donkey anti-goat 488 IgG	1:600	Invitrogen, Cat#A11055 RRID: AB_2534102
Secondary antibodies	Donkey anti-rabbit 488 IgG	1:600	Invitrogen, Cat#A21206 RRID: AB_2535792

Primers		
	Target	Forward/Reverse primer (5′-3′)
SeV genome sequence (qPCR)	SeV	GGATCACTAGGTGATATCGAGC/ACCAGACAAGAGTTTAAGAGATATGTATC
Pluripotency markers (qPCR)	OCT3/4	GGGTTTTTGGGATTAAGTTCTTCA/GCCCCCACCCTTTGTGTT
Pluripotency markers (qPCR)	SOX2	CAAAAATGGCCATGCAGGTT/AGTTGGGATCGAACAAAAGCTATT
Pluripotency markers (qPCR)	NANOG	AGGAAGACAAGGTCCCGGTCAA/TCTGGAACCAGGTCTTCACCTGT
House-keeping genes (qPCR)	GAPDH	GAAGGTCGGAGTCAACGGATT/TGACGGTGCCATGGAATTTG
Targeted mutation analysis/sequencing	PITX2c	ACTGTGGCATCTGTTTGCT/GACGACATGCTCATGGAC

Molecular analysis

The expression of endogenous pluripotency-related genes OCT4, SOX2, and NANOG was assessed by quantitative PCR (qPCR) using the primers reported in Table 2. The expression ratio of the target genes was calculated by using the $2^{-\Delta\Delta C_t}$ method, considering GAPDH as reference gene.

Embryoid body formation assay of pluripotency

For the generation of EBs, UNIBSi003-A cells were resuspended in DMEM/F12 medium supplemented with 20% FBS, 0.1 mM NEAA, 1 mM L-Glutamine, 50 μM 2-mercaptoethanol, 50 U/mL penicillin and 50 mg/mL streptomycin (all from Thermo Fisher Scientific). Seven days later, EBs were transferred onto 0.1% gelatin-coated glass and cultured for additional 14 days. Then, cells were fixed using 4% paraformaldehyde and stained.

Immunofluorescence staining

The cells were incubated with Blocking Buffer (PBS containing 3% Donkey serum, 0.1% Triton X-100) for 60 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 594- and/or Alexa Fluor 488-conjugated secondary antibodies (Thermo Fisher Scientific) were added 1 h at room temperature. Cellular nuclei were counterstained with DAPI. The cells were observed under an inverted fluorescent microscope (Axiovert, Zeiss).

Flow cytometry

The cells were stained with either anti-SSEA4-PE antibody (BD Bioscience) or with anti Tra-1-81 antibody (Millipore), followed by incubations with a biotinylated anti mouse IgM and a PE-conjugated streptavidine. Samples were analyzed using FACSCanto™ flow cytometry (BD Bioscience).

Acknowledgement

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Reference

Christophersen, I.E., Nielsen, J.B., Holst, A.G., Sajadieh, A., Haunsoe, S., Tveit, A., Svendsen, J.H., Olesen, M.S., 2013. The PITX2 variant p.M200V is associated with early-onset lone atrial fibrillation and co-segregates within a family. Eur. Heart J. 34 (suppl_1), 4557.