Stem Cell Research 36 (2019) 101416

Contents lists available at ScienceDirect

Stem Cell Research

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

Lab resource: Stem cell line

Generation of the human induced pluripotent stem cell (hiPSC) line PSMi007-A from a Long QT Syndrome type 1 patient carrier of two common variants in the *NOS1AP* gene

Manuela Mura[a], Federica Pisano[a,b], Manuela Stefanello[a], Monia Ginevrino[c,d], Marina Boni[e], Federica Calabrò[a], Lia Crotti[f,g,h], Enza Maria Valente[c,d], Peter J. Schwartz[f], Paul A. Brink[i], Massimiliano Gnecchi[a,b,j,*]

[a] *Coronary Care Unit and Laboratory of Experimental Cardiology for Cell and Molecular Therapy, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy.*
[b] *Department of Molecular Medicine, Unit of Cardiology, Università degli studi di Pavia, Pavia, Italy.*
[c] *Department of Molecular Medicine, Unit of Genetics, Università degli studi di Pavia, Pavia, Italy.*
[d] *Neurogenetics Unit, Fondazione IRCCS Santa Lucia, Rome, Italy*
[e] *Laboratory of Oncohaematological Cytogenetic and Molecular Diagnostics, Division of Haematology, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy.*
[f] *Istituto Auxologico Italiano, IRCCS, Center for Cardiac Arrhythmias of Genetic Origin and Laboratory of Cardiovascular Genetics, Milan, Italy.*
[g] *Istituto Auxologico Italiano, IRCCS, Department of Cardiovascular, Neural and Metabolic Sciences, San Luca Hospital, Milan, Italy.*
[h] *Department of Medicine and Surgery, University of Milano-Bicocca, Milan, Italy*
[i] *Department of Internal Medicine, University of Stellenbosch, Tygerberg, South Africa.*
[j] *Department of Medicine, University of Cape Town, Cape Town, South Africa*

ABSTRACT

We generated human induced pluripotent stem cells (hiPSCs) from a symptomatic Long QT Syndrome (LQTS) type 1 patient, belonging to a South African (SA) founder population segregating the heterozygous mutation c.1022C > T p.A341V on the *KCNQ1* gene. The patient is also homozygous for the two minor variants rs4657139 and rs16847548 on the *NOS1AP* gene, associated with greater risk for cardiac arrest and sudden death in LQTS mutation carriers of the founder population. hiPSCs, obtained using four retroviruses encoding the reprogramming factors OCT4, SOX2, cMYC and KLF4, display pluripotent stem cell characteristics, and can be differentiated into spontaneously beating cardiomyocytes (hiPSC-CMs).

Resource table

Unique stem cell line identifier	PSMi007-A
Alternative name of stem cell line	SA13.5-iPS
Institution	Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
Contact information of distributor	Massimiliano Gnecchi, m.gnecchi@unipv.it
Type of cell line	hiPSC
Origin	human
Additional origin info	Age: 64 Gender: female Ethnicity: Caucasian
Cell source	Dermal fibroblasts
Clonality	Clonal
Method of reprogramming	Retroviruses encoding for the human cDNAs of OCT4, SOX2, cMYC, KLF4
Genetic modification	Yes
Type of modification	Congenital
Associated disease	Long QT Syndrome type 1 (OMIM #192500)
Gene/locus	1022C > T mutation on KCNQ1 (NM_000218.2) rs4657139 variant (CM000663.2:g.162060117A > T) rs16847548 variant (CM000663.2:g.162065484 T > C)
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/Constitutive system	N/A
Date archived/stock date	13/1/2016
Cell line repository/bank	https://hpscreg.eu/cell-line/PSMi007-A
Ethical approval	The study has been approved by the Ethics Committee of the University of Stellenbosch, South Africa, on the 4 March 2013, protocol number N13/01/002. We obtained patient written informed consent for both skin biopsy procedure and conservation of biological samples.

* Corresponding author at: Fondazione IRCCS Policlinico S. Matteo, University of Pavia, Pavia, Italy.
E-mail address: m.gnecchi@unipv.it (M. Gnecchi).

https://doi.org/10.1016/j.scr.2019.101416
Received 28 January 2019; Received in revised form 27 February 2019; Accepted 5 March 2019
Available online 06 March 2019

Resource utility

iPSCs and iPSC-CMs can be used to model LQTS and drug testing (Mehta et al., 2018; Gnecchi et al., 2017; Mura M et al. 2017). PSMi007-A cell line will be useful for modelling LQTS type 1, to identify cellular mechanisms by which NOS1AP rs4657139 and rs16847548 variants influence the clinical severity in carriers of the KCNQ1-A341V mutation, and to test personalized therapeutics.

Resource details

The PSMi007-A cell line was generated by reprogramming of dermal fibroblasts isolated from a 64 years old woman affected by Long QT Syndrome type 1 (LQT1). LQTS is an autosomal dominant inherited disease characterized by the prolongation of cardiac repolarization, which is quantified as the duration of the QT interval on the surface electrocardiogram (ECG). The repolarization defect predisposes to "Torsades de Pointes" (TdP) type of ventricular tachycardia, often causing syncope or sudden cardiac death (SCD). LQT1 is the most common LQTS sub-type, accounting for ~40–50% of all LQTS cases and is caused by mutations in the *KCNQ1* gene, encoding for the α-subunit of the voltage-dependent potassium channel responsible for the delayed rectifier potassium current (I_{Ks}), one of the repolarization currents in the heart.

The patient belongs to a SA LQT1 founder population extensively characterized by our group and segregating the KCNQ1-A341V mutation in heterozygosis (Brink et al., 2005). In these patients, two single nucleotide polymorphisms (SNPs), rs4657139 and rs16847548, in the *NOS1AP* gene are associated with almost doubled risk of sudden death (malignant phenotype) (Crotti et al., 2009). This finding is of particular interest since the physiological role of NOS1AP protein suggests that a cause-effect relationship may underlie the linkage between these SNPs and the disease severity.

Fibroblasts were reprogrammed by retroviral infection of OCT4, SOX2, KLF4 and c-MYC. The obtained hiPSCs were maintained on feeders. Both patient's fibroblasts and the derived hiPSCs present the disease causing mutation on the *KCNQ1* gene, as proven by DNA sequencing (Fig. 1A. *Left panel. The KCNQ1 coding sequence -CDS- used as a reference is the NCBI sequence NM_000218.2*), are homozygous for rs4657139 and rs16847548 minor alleles (Fig. 1A. *Right panel. MAF = minor allele frequency in the SA founder population*), and an identical DNA profile at 7 polymorphic loci, as shown by Short tandem Repeat (STR) analysis (available with the authors). Moreover the DNA karyotyping at passage 13 revealed normal female karyotype (46, XX) (Fig. 1B). PSMi007-A displays ES-like morphology, uniformly expresses the human ES surface antigens Tumor Related Antigen-1-60 (TRA-1-60), Stage Specific Embryonic Antigen-3 and -4 (SSEA-3, SSEA-4), and shows alkaline phosphatase (AP) activity (Fig. 1C). Likewise, it expresses the pluripotent markers NANOG, OCT4, SOX2 (Fig. 1C and D), REX1, GDF3, DPPA2, and NODAL (Fig. 1D). RT-PCR analysis in Fig. 1E shows no expression of the four viral transgenes (Tg) in naïve fibroblasts (HDF), clear expression of Tg OCT4, SOX2, KLF4 and cMYC in fibroblasts five days after transduction (OSKM) and silencing of the four Tg in PSMi007-A at passage 7.

PSMi007-A spontaneously forms embryoid bodies (EBs) able to differentiate into cells belonging to the three germ layers: endoderm (alpha-fetoprotein - AFP), mesoderm (alpha smooth muscle actin-αSMA) and ectoderm (tubulin beta III - Tuj) (Fig. 1F). Most importantly, we have successfully differentiated this LQT1 cell line into cardiomyocytes displaying spontaneous beating activity, and expressing the sarcomeric proteins alpha-actinin (α-SA) and troponin T (TnT) (Fig. 1G, *the magnifications show areas of cross-striation*). We also verified the absence of mycoplasma contamination in our PSMi007-A line (Fig. 1H).

Materials and methods

hiPSC generation

The detailed protocol is reported in the Supplemental Methods section.

Skin fibroblasts were reprogrammed using four retroviruses pMXs-hOCT3/4 (Addgene #17217), pMXs-hSOX-2 (Addgene #17218), pMXs-hcMYC (Addgene #17220) and pMXs-hKLF4 (Addgene #17219), that were packaged in the 293 T cell line (Clontech), using the packaging vector pCL-Eco (Addgene #12371). Emerging iPSC clones were manually picked, individually placed into a separate cell culture well and expanded on a feeder-layer of mitotically-inactivated mouse embryonic fibroblasts (iMEF), in DMEM/F12 (Gibco) supplemented with 20% Knockout Serum Replacement (KO-SR), 2 mM L-glutamine, 50 U/ml penicillin, 50 U/ml streptomycin, 1% Non-Essential Amino Acids (NEAA), 0.1 mM beta-mercaptoethanol and 10 ng/ml basic Fibroblast Growth Factor (bFGF) (all purchased from Gibco), in a humidified incubator, at 37 °C, 5% CO_2. Passaging was performed every 5–7 days, at 1:3 split ratio, using a dissociation buffer composed by 1 mg/ml collagenase IV (Invitrogen), 0,25% trypsin (Gibco), 20%KO-SR (Gibco), 1 mM $CaCl_2$ all diluted in PBS 1 × (Table 1).

Mutation analysis

Genomic DNA was extracted from hiPSCs and their parental fibroblasts with QIAamp DNA Blood Mini kit (Qiagen). *KCNQ1* exon 6, rs4657139 and rs16847548 genomic regions were amplified with the Phire Green Hot Start II PCR Master Mix (Thermo Scientific) and Mastercycler EPGradient S (Eppendorf) (see Table 2 for primer sequences and product sizes). Cycle parameters were: 30 s at 98 °C, (5 s at 98 °C, 5 s at 56 °C, 30 s at 72 °C) x 35 times, 60 s at 72 °C.The resulting amplicons were purified and sequenced (Lightrun service - GATC Biotech AG – Germany).

STR analysis

STR analysis was carried out using PowerPlex® CS7 System (Promega) kit, following the manufacturer's protocol. Fragments were run on a 3130xl capillary sequencer (Applied Biosystems). Genotypes were assigned using GeneMarker software (SoftGenetics).

Karyotyping

hiPSCs were blocked at metaphase by exposure to 10 μg/ml demecolcine solution (Sigma Aldrich) for 3 h, and then lysed with a hypotonic solution (0.075 M KCl), and fixed with fresh Carnoy's Fixative (3:1 ratio of methanol:glacial acetic acid). Karyotyping was performed using the Giemsa trypsin G-banding (GTG-banding) technique. Whenever possible, we screened at least 20 metaphases, and 6 of them fully karyotyped. Chromosome identification and karyotype description were made in accordance with the International System for Chromosome Nomenclature (ISCN, 2016).

Immunocytochemistry

hiPSCs and their derivatives were grown on glass coverslips, and then fixed for 15 min in 4% paraformaldehyde (Affymetrix USB), permeabilized with 0.1% Triton X-100 (Sigma Aldrich) for 5 min, and

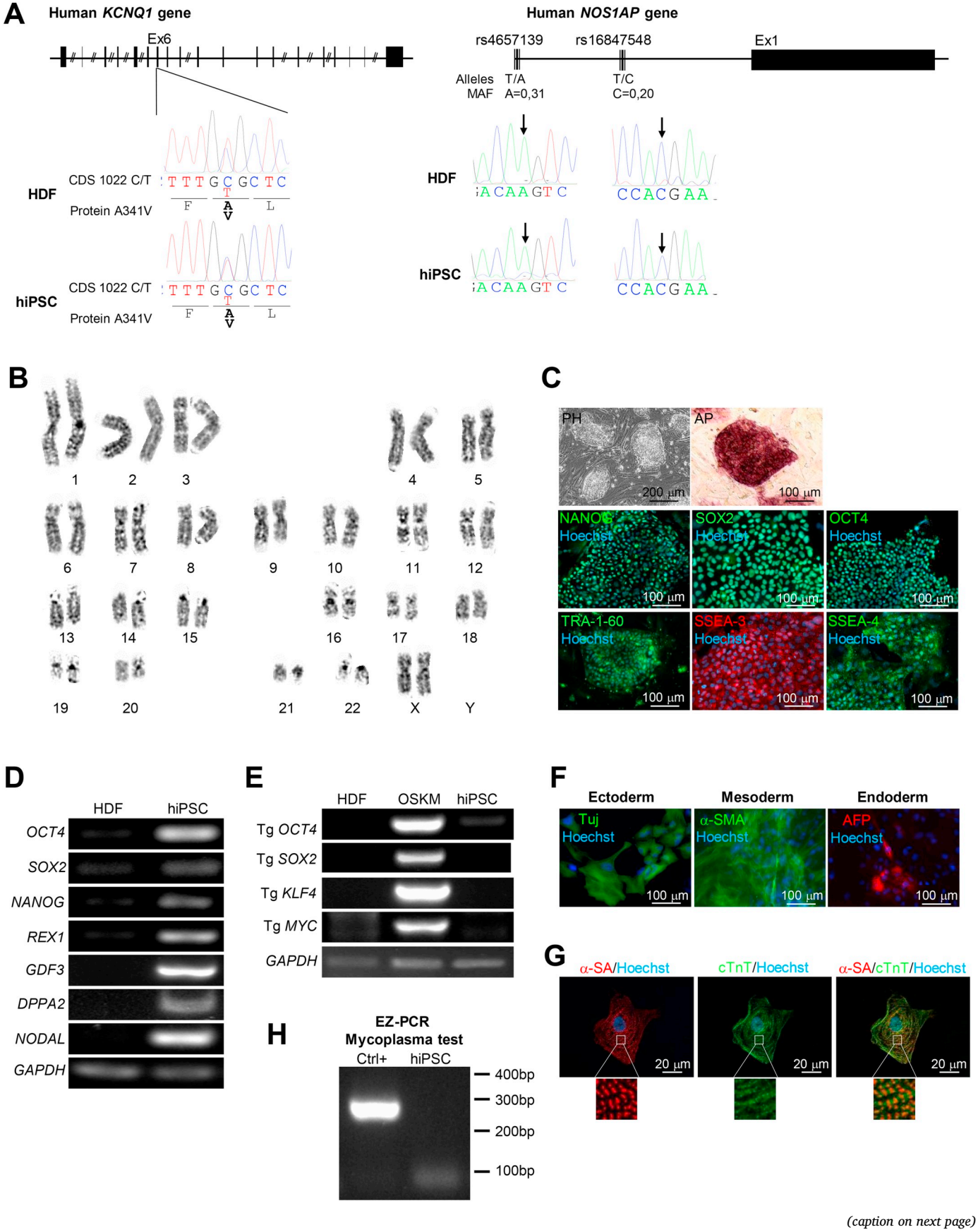

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Fig. 1. Characterization of the PSMi007-A cell line. A. Top: schematic representation of *KCNQ1* and *NOS1AP* genes (exons are vertical lines/boxes). The KCNQ1 coding sequence (CDS) used as a reference is the NCBI sequence NM_000218.2. MAF is the minor allele frequency in the SA founder population. Bottom: DNA sequencing results showing the mutation 1022 C/T in the *KCNQ1* gene in heterozygosis, and the rs4657139 and rs16847548 minor alleles in homozygosis, in both patient-derived dermal fibroblasts (HDF) and PSMi007-A cell line (hiPSC). **B.** Karyotype analysis of PSMi007-A (300 G-bandings) showing normal female karyotype (46, XX). **C.** Top left: phase contrast image showing PSMi007A morphology (PH). Top right: alkaline phosphatase colorimetric staining (AP). Bottom panels: immunofluorescence stainings showing uniform expression of the indicated markers of pluripotency in the PSMi007-A. Nuclei were counterstained with Hoechst 33258 (Hoechst, blue). **D.** RT-PCR analysis showing expression of the indicated markers of pluripotency in PSMi007-A (hiPSC) compared with its parental fibroblasts (HDF). **E.** RT-PCR analysis showing no expression of the four viral transgenes (Tg) in naïve fibroblasts (HDF), expression of Tg OCT4, SOX2, KLF4 and cMYC five days after transduction (OSKM) and silencing of the four Tg in PSMi007-A at passage 7. **F.** Immunofluorescence staining for markers of the 3 germ layers in iPSC-derived EBs: neuronal class tubulin beta III (Tuj) for ectoderm, smooth muscle actin (SMA) for mesoderm, and alpha Fetoprotein (AFP) for endoderm. **G.** Co-immunofluorescence staining for the alpha-sarcomeric actinin (α-SA, red) and troponin T (TnT, green) in cardiomyocytes differentiated from the PSMi007-A. Nuclei were counterstained with Hoechst. Magnifications show areas of cross-striations. **H.** EZ-PCR test showing the absence of mycoplasma contamination in PSMi007-A. Ctrl + is the positive PCR control provided by the kit.

Table 1
Characterization and validation of PSMi007-A cell line.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel C
Phenotype	Qualitative analysis	Positive immunostaining for the pluripotency markers OCT4, NANOG, SOX2, TRA-1-60, SSEA-3, SSEA-4	Fig. 1 panel C
		Positive staining for the alkaline phosphatase	Fig. 1 panel C
	RT-PCR	Expression of the pluripotency markers OCT3/4, SOX2, NANOG, REX1, GDF3, DPPA2, NODAL	Fig. 1 panel D
Genotype	Karyotype (300 G-banding) and resolution	46XX, Resolution 450–500	Fig. 1 panel B
Identity	Microsatellite PCR (mPCR)	Not performed	Not available
	STR analysis	7 sites tested for iPSC, all sites matched with donor HDF STR profile	Available with the author
Mutation analysis	Sequencing	Heterozygous for the mutation c. 1022C > T p.A341V on the KCNQ1 gene. Homozygous for rs4657139 and rs16847548 minor alleles	Fig. 1 panel A
Microbiology and virology	Mycoplasma	Mycoplasma testing by RT-PCR. Negative	Fig. 1 panel H
Differentiation potential	Embryoid body formation	The EBs expressed neuronal class tubulin beta III (Tuj) (ectoderm), smooth muscle actin (SMA) (mesoderm), and alpha Fetoprotein (AFP) (endoderm).	Fig. 1 panel F
	Differentiation into cardiomyocytes	The iPSC-derived cardiomyocytes expressed the cardiac sarcomeric proteins alpha-sarcomeric actinin (α-SA) and troponin T (TnT)	Fig. 1 panel G
Donor screening	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	Not available
Genotype additional info	Blood group genotyping	Not performed	Not available
	HLA tissue typing	Not performed	Not available

blocked in 1% bovine serum albumin (BSA, Sigma Aldrich) for 1 h at room temperature (RT). Then, they were incubated for 1 h at RT with the primary antibody (Table 2) diluted in blocking solution, washed three times, and incubated for 1 h at RT with an appropriate secondary antibody (Table 2). Finally, the cells were stained with 1 μg/ml of Hoechst 33258 (Sigma Aldrich). Images were acquired using the Carl Zeiss fluorescence microscope Observer.Z1 equipped with the Apotome system and AxioVision 6.0 software (Zeiss GmbH, Gottingen, Germany).

AP assay

AP was detected by using the Alkaline Phosphatase Staining kit II (00–0055 Stemgent).

RT-PCR

Total RNA was purified using TRIzol (ThermoFisher Scientific) and quantified using Nanodrop ND-1000 spectrophotometer (Celbio). 500 ng of purified RNA was reverse transcribed into cDNA using the Superscript IV Reverse Transcriptase (ThermoFisher), following manufacturer's instructions. 1 μl of the RT reaction was amplified by PCR with the Phire Green Hot Start II PCR Master Mix (Thermo Scientific) and primers in Table 2. Cycle parameters were: 30 s at 98 °C, (5 s at 98 °C, 5 s at 56 °C, 30 s at 72 °C) x 26–33 times, 60 s at 72 °C. As a thermocycler for both RT and PCR, we used the Mastercycler EP-Gradient S (Eppendorf).

EB formation

hiPSCs were detached using our dissociation buffer (see "hiPSC generation" paragraph), and grown for 7 days in non-adherent conditions in a modified iPS medium deprived of bFGF and containing 20% FBS instead of KO-SR. Forming EBs were then transferred to gelatin-coated dishes to allow differentiation in adhesion for additional 7 days in the same medium. Finally, the cells were processed for immunostaining of the three germ layers as described above.

Cardiac differentiation

Cardiac differentiation was induced using the PSC Cardiomyocyte Differentiation Kit (ThermoFisher).

Mycoplasma test

For the detection of mycoplasma in cell culture we used the EZ-PCR Mycoplasma Test Kit (Biological Industries).

Table 2
Reagents details.

Antibodies used for immunocytochemistry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit anti NANOG	1:200	Stemgent *Cat#* 09–0020, *RRID:* AB_2298294
	Mouse anti OCT3/4 (C-10)	1:500	SCBT *Cat#* sc-5279, *RRID:* AB_628051
	Mouse anti SOX2	1:500	R&D Systems *Cat#* MAB2018, *RRID:* AB_358009
	Mouse anti TRA-1-60	1:100	Stemgent *Cat#* 09–0010, *RRID:* AB_1512170
	Rat anti SSEA-3	1:100	Millipore *Cat#* MAB4303, *RRID:* AB_177628
	Mouse anti SSEA-4	1:100	Stemgent *Cat#* 09–0006, *RRID:* AB_1512169
Differentiation Markers (EBs)	Mouse anti neuronal class tubulin beta III (Tuj)	1:500	Covance *Cat#* MMS-435P, *RRID:* AB_2313773
	Mouse anti alpha smooth muscle actin (α-SMA)	1:1000	Millipore *Cat#* CBL171, *RRID:* AB_2223166
	Mouse anti alpha-fetoprotein	1:500	Millipore *Cat#* SCR030, *RRID:* AB_597591
Cardiac Markers	Rabbit anti Troponin T	1:250	Abcam *Cat#* 45932, *RRID:* AB_ AB_956386
	Mouse anti alpha actinin	1:800	Sigma Aldrich *Cat#* A7811, *RRID:* AB_476766
Secondary antibodies	Alexa-Fluor® 488 Goat anti-rabbit IgG	1:500	ThermoFisher Cat# A11008, *RRID:*AB_143165
	Alexa-Fluor® 594 Goat anti-rat IgM	1:500	ThermoFisher Cat# A21213, *RRID:* AB_11180463
	Alexa-Fluor® 488 Goat anti-mouse IgG	1:500	ThermoFisher Cat# A11001, *RRID:* AB_2534069
	Alexa-Fluor® 546 Goat anti-mouse IgG	1:500	ThermoFisher Cat# A11003, *RRID:* AB_141370

Primers		
	Target	Forward/Reverse primer (5′-3′)
Targeted mutation analysis/sequencing	KCNQ1 Exon 6	Fw: 5′- tggctgaccactgtccctct −3′
	195 bp	Rev.: 5′- ccccaggaccccagctgtccaa −3′
	rs4657139	Fw: 5′- tgagtacacgacagaggtgg −3′
	623 bp	Rev.: 5′- ggagacacagaaaatgtcacaggg −3′
	rs16847548	Fw: 5′- aggggaacttaaaccgtgcc −3′
	579 bp	Rev.: 5′- agcgccctctatcaccaatg −3′
Pluripotency Markers (RT-PCR)	OCT4	Fw: 5′-gtactcctcggtcccctttcc-3′
	168 bp	Rev.: 5′-caaaaaccctggcacaaact-3′
	SOX2	Fw: 5′-acaccaatcccatccacact-3′
	273 bp	Rev.: 5′-tttttcgtcgcttggagact-3′
	NANOG	Fw: 5′-ttccttcctccatggatctg-3′
	213 bp	Rev.: 5′-tctgctggaggctgaggtat-3′
	REX1	Fw: 5′-cagatcctaaacagctcgcagaat-3′
	306 bp	Rev.: 5′-gcgtacgcaaattaaagtccaga-3′
	GDF3	Fw: 5′-cttatgctacgtaaaggagctggg-3′
	631 bp	Rev.: 5′-gtgccaacccaggtcccggaagtt-3′
	DPPA2	Fw: 5′-ccgtccccgcaatctccttccatc-3′
	606 bp	Rev.: 5′-atgatgccaacatggctcccggtg-3′
	NODAL	Fw: 5′-gggcaagaggcaccgtcgacatca-3′
	234 bp	Rev.:5′-gggactcggtggggctggtaacgtttc-3′
House-Keeping Genes (RT-PCR)	GAPDH	Fw 5′-catgttccaatatgattccaccc-3′
	112 bp	Rev. 5′-gggatctcgctcctggaagat-3′
Retroviral transgenes	Oct4 cDNA on pMXs-hOCT3/4	Fw: 5′-ccccagggccccattttggtacc-3′
	339 bp	
	Sox2 cDNA on pMXs-hSOX-2	Fw: 5′-ggcacccctggcatggctcttggctc-3′
	496 bp	
	cMyc cDNA on pMXs-hcMYC	Fw: 5′-caacaaccgaaaatgcaccagccccag-3′
	542 bp	
	Klf4 cDNA on pMXs-hKLF4	Fw: 5′-acgatcgtggccccggaaaaggacc-3′
	518 bp	
	pMX viral vector	Rev: 5′-ccctttttctggagactaaataaa-3′

Acknowledgments

This work was supported by the Leducq Foundation for Cardiovascular Research [18CVD05] ‘Towards Precision Medicine with Human iPSCs for Cardiac Channelopathies’, and by the Ministero Italiano della Salute, "Ricerca Corrente" projects numbers 08064017 and 08064018.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101416.

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