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Generation of heterozygous (MRli003-A-3) and homozygous (MRli003-A-4) TRPM4 knockout human iPSC lines

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A B S T R A C T

TRPM4 is a Ca^{2+}-activated channel mediating the transport of monovalent cations across the cell membrane. Mutations in the *TRPM4* gene have been associated with cardiac arrhythmias in humans. Using CRISPR/Cas9 gene editing technology, we established two *TRPM4* knockout human iPSC lines – one heterozygous (MRli003-A-3) and one homozygous (MRli003-A-4) – by inserting a frameshift mutation in exon 2 of the *TRPM4* gene. Both lines maintained pluripotency, a normal karyotype, parental cell morphology, and the ability to differentiate into the three germ layers.

1. Introduction

1.1. Resource utility

As*TRPM4* mutations have been implicated in inherited cardiac arrhythmias in humans (Amarouch et al., 2020), these heterozygous and homozygous *TRPM4* knockout iPSC lines may be useful to study the underlying mechanisms.

2. Resource table

Unique stem cell line identifier	Cell line 1: MRli003-A-3 Cell line 2: MRli003-A-4
Alternative name(s) of stem cell lines	Cell line 1: TRPM4+/− Cell line 2: TRPM4−/−
Institution	Klinik und Poliklinik Innere Medizin I, Klinikum rechts der Isar – Technical University of Munich, Munich, Germany
Contact information of the reported cell line distributor	amoretti@mytum.de
Type of cell lines	iPSCs
origin	Human, MRli003-A hiPSCs described in Moretti et al. 2020, Nature Medicine
Additional origin info	Age: N/A Sex: Male Ethnicity: Caucasian
Cell Source	Peripheral Blood Mononuclear Cell
Method of reprogramming	Non-integrating sendai virus OCT3/4, SOX2, KLF4 and MYC
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	N/A
Cell culture system used	Essential 8™
Type of Genetic Modification	Induced mutation: Cell line 1_ MRli003-A-3: frame shift in one allele of exon 2 of *TRPM4* gene Cell line 2_ MRli003-A-4: frame shift in two alleles of exon 2 of *TRPM4* gene
Associated disease	N/A
Gene/locus	*TRPM4* gene/19q13.33
Method of modification/site-specific nuclease used	CRISPR/Cas9
Site-specific nuclease (SSN) delivery method	Plasmid transfection
All genetic material introduced into the cells	Cas plasmid
Analysis of the nuclease-targeted allele status	Sanger Sequencing
Method of the off-target nuclease activity surveillance	Top off-target site analyzed by PCR/ sequencing in genomic exons
Name of transgene or resistance	N/A N/A

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(*continued*)

Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	
Inducible/constitutive system	N/A
Date archived/stock date	July 15, 2021
Cell line repository/bank	Cell line 1: https://hpscreg.eu/cell-line/MRIi003-A-3 Cell line 2: https://hpscreg.eu/cell-line/MRIi003-A-4
Ethical/GMO work approvals	The choice of appropriate human donors, the procedures for taking peripheral blood, isolation of mononuclear cells from blood, generation of iPSCs and their use in further scientific investigations were performed under the positive votes of the Ethics Committee of the Faculty of Medicine (TUM, approval number: 2109/08). All study participants gave informed consent prior to entry into the study.

2.1. Resource details

The *TRPM4* gene encodes a transient receptor potential melastatin-like subfamily member 4 (TRPM4) protein, which is an intracellular calcium-activated nonselective channel permeable to monovalent cations (Amarouch et al., 2020; Wang et al., 2018). While it is regulated by intracellular Ca^{2+}, it is not permeable to Ca^{2+}. TRPM4 is detected ubiquitously and is abundantly expressed in cardiac cells. Recent evidence suggests that it plays an important role in cardiac excitability and automaticity (Amarouch et al., 2020; Wang et al., 2018).

Here, using CRISPR/Cas9 technology, we generated heterozygous (MRIi003-A-3) and homozygous (MRIi003-A-4) *TRPM4* knockout human iPSC lines with a single guide RNA (sgRNA) targeting *TRPM4* exon 2 (Fig. 1A, Table 1). After editing, sequencing revealed a heterozygous 4 nt (CGAC) deletion and a 2 nt (TA) insertion in the MRIi003-A-3 line and a homozygous 1 nt (A) insertion in the MRIi003-A-4 line, which caused a premature stop codon in both cases (Fig. 1B, Fig. S1A). Both lines displayed typical iPSC colony formation (Fig. 1C) and expressed the pluripotency markers OCT3/4 and NANOG (Fig. 1D). Moreover, mRNA expression of the pluripotency markers *OCT3/4, SOX2, REX1, NANOG,* and *TDGF1* was significantly higher in these lines compared to fibroblasts, as detected by qRT-PCR (Fig. S1B). Flow cytometry analysis showed that over 98% of cells expressed both surface pluripotency markers TRA-1–60 and SSEA4 (Fig. 1E). Both lines successfully differentiated towards all three germ layers, as demonstrated by qRT-PCR analysis of specific markers of endoderm (*FOXA2, SOX17, CXCR4,* and *GATA4*), mesoderm (*TBXT, DES, ACTA2, SCL,* and *CDH5*), and ectoderm (*PAX6, KRT14, NCAM1, TH,* and *GABRR2*) (Fig. 1F). Both edited lines also presented a normal karyotype (46, XY) (Fig. 1G) and were free of mycoplasma contamination (Fig. S1C). No mutations were found at the coding region of the *PI4KA* gene, the only coding locus exhibiting a high off-target editing score for the sgRNA used (Fig. S1D).

As *TRPM4* is not expressed in undifferentiated iPSCs, we subjected both lines to a Wnt-based cardiac induction protocol, which showed that they can be efficiently differentiated into $cTNT^{+}$ α-$actinin^{+}$ cardiomyocytes (CMs) (Fig. S1E, Fig. 1H). This allowed us to confirm that the indels introduced in exon 2 of the *TRPM4* gene led to the premature termination of protein translation, as Western blot analysis showed decreased TRPM4 protein levels in cardiomyocytes derived from the MRIi003-A-3 line compared to unmodified cells and complete absence of the protein in cardiomyocytes from the MRIi003-A-4 line (Fig. 1I).

3. Materials and methods

3.1. hiPSC culture

hiPSCs were cultured on Geltrex-coated (Thermo Fisher Scientific, A14133-02) plates in Essential 8 medium (Thermo Fisher Scientific, A1517001) containing 0.5% Penicillin/Streptomycin (Thermo Fisher Scientific, 15140–122) at 37 °C, 5% CO_2. Cells were passaged at a ratio of 1:14 every 4–5 days using 0.5 mM EDTA (Thermo Fisher Scientific, AM9260G). After passaging, the medium was supplemented with 10 μM Thiazovivin (Sigma-Aldrich, SML1045) for 24 h.

3.2. Cloning of targeting constructs

A sgRNA targeting exon 2 of the *TRPM4* gene was designed and evaluated with CRISPOR (http://crispor.tefor.net). It was then cloned into a pSpCas9(BB)-2A-Puro (PX459) V2.0 plasmid (Addgene, 62988), referred to as pSpCas9(BB)-TRPM4-KOgRNA-2A-Puro. Cloning steps were performed according to Ran et al. (2013).

3.3. 4D Nucleotransfection of hiPSCs

Nucleotransfection was performed on 10^6 cells following the Lonza Amaxa 4D Nucleofector basic protocol for human stem cells. hiPSCs were transfected with 5 μg pSpCas9(BB)- TRPM4-KOgRNA-2A-Puro plasmid and reseeded in Matrigel-coated (BD, 354277) 24 well-plates in mTeSR1 (Stemcell Technologies, 05854) containing 10 μM Thiazovivin. 24 h later, 0.2 μg/ml puromycin (Calbiochem, 540411) was added for 24 h. Another 1–2 days later, cells were dissociated with Accutase (Thermo Fisher Scientific, A11105-01) and replated for single clone expansion.

3.4. Immunocytochemistry

Immunocytochemistry was performed as previously described (Dorn et al., 2018) with the antibodies listed in Table 2.

3.5. Western blotting

Cell lysates of iPSC-derived day 15 cardiomyocytes were prepared with RIPA buffer (Sigma-Aldrich, R0278) containing a protease inhibitor (Roche, 11836170001). Western blotting was performed using standard protocols. Antibodies are listed in Table 2.

3.6. Flow cytometry

iPSCs were dissociated with Accutase and 10^6 cells were stained with TRA-1–60-PE or the corresponding isotype antibody (Table 2). Data were acquired at a Gallios flow cytometer (Beckman Coulter, Germany) and analyzed with Kaluza software version 1.2.

3.7. Quantitative real-time PCR (qRT-PCR)

Total mRNA was isolated with the Absolutely Microprep RNA kit (Agilent, 400805) and cDNA was prepared with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, 4368813). qRT-PCR was performed with the Power SYBR Green PCR Master Mix (Applied Biotechnologies, 4367659) and the primers listed in Table 2 using a 7500 Fast Real-Time PCR instrument (Applied Biosystems, Germany). Gene expression levels were normalized to *GAPDH*.

3.8. Mycoplasma detection

Mycoplasma detection was performed with the MycoAlert PLUS Mycoplasma Detection Kit (Lonza, LT07-703).

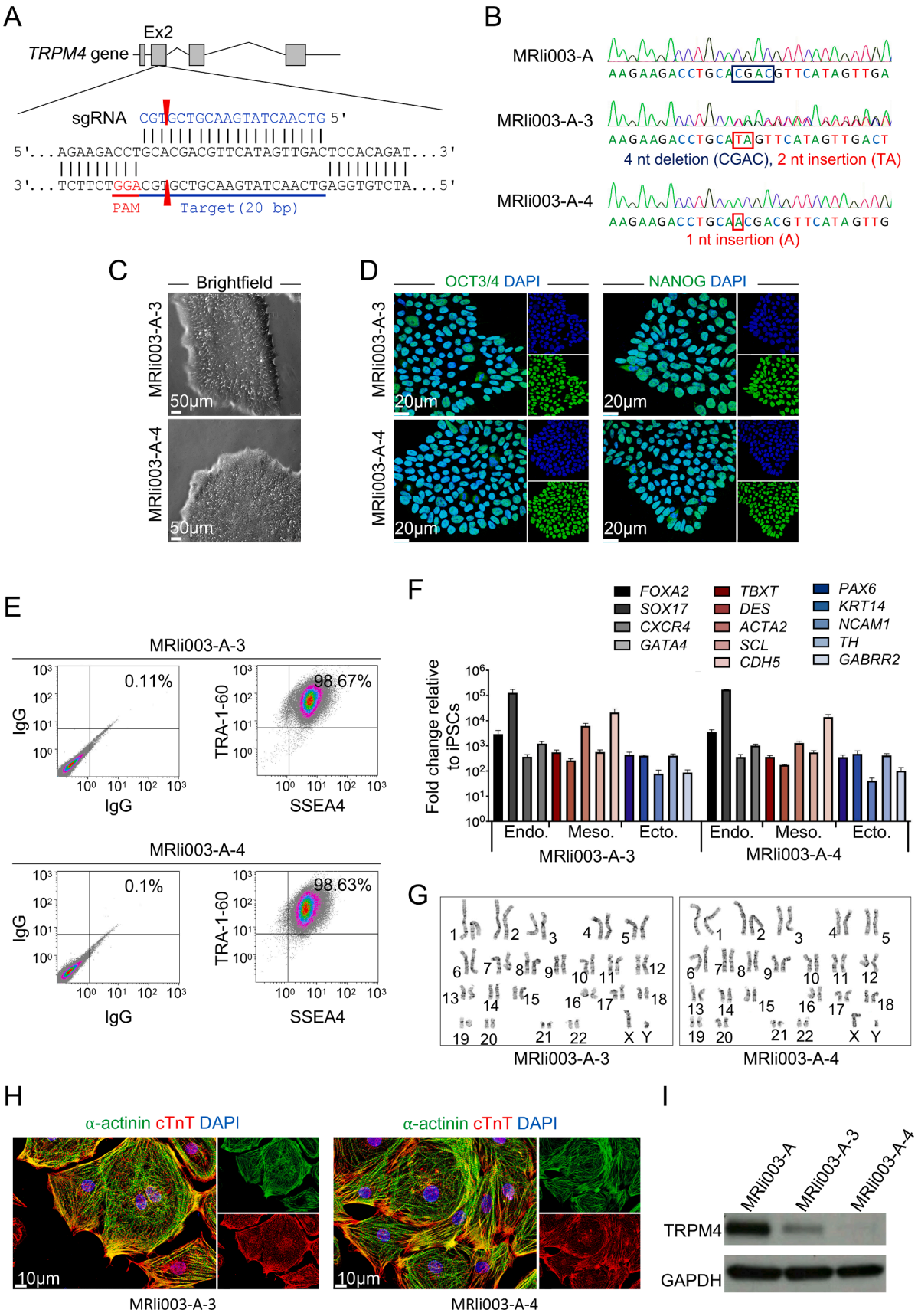

Fig. 1. Generation and validation of the *TRPM4* knockout iPSC lines MRli003-A-3 and MRli003-A-4.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Brightfield imaging	Normal morphology	Fig. 1C
Pluripotency status evidence for the described cell line	Immunocytochemistry	OCT3/4 and NANOG staining	Fig. 1D
	Flow cytometry	98% Double positive of SSEA4 and TRA-1–60 for MRli003-A-3 and MRli003-A-4	Fig. 1E
	RT-qPCR	mRNA level of *OCT3/4*, *SOX2*, *REX1*, *NANOG* and *TDGF1*	Fig. S1B
Karyotype	Karyotype (G-banding) and resolution	MRli003-A-1: 46, XY; Resolution 450–525 bands MRli003-A-2: 46, XY; Resolution 425–550 bands	Fig. 1G
Genotyping for the desired genomic alteration/ allelic status of the gene of interest	PCR across the edited site/ Sequencing	Both MRli003-A-3 and MRli003-A-4 determined by PCR and confirmed by Sanger sequencing	Fig. 1B, Fig. S1A
	Transgene-specific PCR	N/A	N/A
Verification of the absence of random plasmid integration events	PCR/Southern	N/A	N/A
Parental and modified cell line genetic identity evidence	STR analysis	Performed by Eurofins MWG Operon company	Supplementary file, submitted in the archive with journal
Mutagenesis /genetic modification outcome analysi	Sequencing (genomic DNA PCR or RT-PCR product)	Sanger sequencing tracks	Fig. 1B
	PCR-based analyses	Not performed	Not performed
	Western blotting (for knockouts, KOs)	TRPM4 protein levels were determined in both MRli003-A-3 and MRli003-A-4	Fig. 1I
Off-target analysis	PCR across top predicted off-target sites and sequencing	Sanger sequencing	Fig. S1D
Specific pathogen-free status	Mycoplasma	Biochemical luminescence MycoAlert™ Plus Mycoplasma Detection Kit, Lonza, Negative	Fig. S1C
Multilineage differentiation potential	Directed differentiation	Three germ layer formation: germ layer specific gene expression checked by RT-qPCR	Fig. 1F
Donor screening	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype - additional histocompatibility info	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Rabbit anti-OCT3/4	1:200	Abcam Cat# ab19857, RRID:AB_445175
	Rabbit anti-NANOG	1:200	Abcam Cat# ab21624, RRID:AB_446437
	Human anti-TRA-1–60-PE	1:10	Miltenyi Biotec Cat# 130–100-347, RRID:AB_2654227
	Human anti-SSEA4-FITC	1:10	Miltenyi Biotec Cat# 130–098-371, RRID:AB_2653517
Cardiomycyte specific markers	Rabbit anti-cTNT	1:500	Abcam Cat# ab92546, RRID:AB_10585593
	Mouse anti-α-actinin	1:200	Sigma-Aldrich Cat# A7811, RRID:AB_476766
Isotype control	REA Control (S)-PE-Vio615	1:10	Miltenyi Biotec Cat# 130–107-146, RRID:AB_2661694
	REA Control (S)-FITC	1:10	Miltenyi Biotec Cat# 130–104-610, RRID:AB_2661688
TRPM4 specific antibody	Rabbit anti-TRPM4	1:200	Custom-made by Prof. Veit Flockerzi, Department of Pharmacology and Toxicology, Saarland Uniserity, Germany
	Rabbit anti-GAPDH	1:2000	Cell signaling Cat# 2118, RRID:AB_561053
Secondary antibodies	Goat Anti-Rabbit Alexa Fluor 488 IgG Goat Anti-Mouse Alexa Fluor 594 IgG	1:500	Invitrogen Cat# A11008, RRID:AB_143165
	Anti-rabbit IgG HRP	1:500	Invitrogen Cat# A-11005, RRID:AB_141372
		1:10000	Sigma-Aldrich Cat# A0545, RRID:AB_257896
Nuclear stain	Hoechst 33,258	1 μg/ml	Sigma-Aldrich Cat# 94,403
Site-specific nuclease			
Nuclease information	WT S. pyogenes Cas9 (SpCas9)	Ran et al Nat Protoc. 2013 Nov;8(11):2281–308	
Delivery method	Nucleotransfection	Lonza; 4D-NucleofectorTM core Unit + X Unit (AAF-1002B + AAF-1002X)	
Selection/enrichment strategy	Antibiotic selection	Puromycin	
Primers and Oligonucleotides used in this study			
	Target	Forward/Reverse primer (5′-3′)	
Pluripotency Markers	*OCT3/4*	GACAGGGGGAGGGGAGGAGCTAGG/ CTTCCCTCCAACCAGTTGCCCCAAAC	
	SOX2	GGGAAATGGGAGGGGTGCAAAAGAGG/ TTGCGTGAGTGTGGATGGGATTGGTG	
	NANOG	TGCAAGAACTCTCCAACATCCT/ATTGCTATTCTTCGGCCAGTT	
	REX1	ACCAGCACACTAGGCAAACC/TTCTGTTCACACAGGCTCCA	
	TDGF1	CCCAAGAAGTGTTCCCTGTG/ACGTGCAGACGGTGGTAGTT	
Three germ layer Markers	*FOXA2*	CCGACTGGAGCAGCTACTATG/TGTACGTGTTCATGCCGTTC	
	SOX17	ACGCCGAGTTGAGCAAGA/GCGGCCGGTACTTGTAGTT	
	CXCR4	CCCTCCTGCTGACTATTCCC/TAAGGCAACCATGTGTGC	

(*continued on next page*)

Table 2 (*continued*)

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
	GATA4		GGCCTGTCATCTCACTACGG/ATGGCCAGACATCGCACT
	TBXT		TGTTTATCCATGCTGCAATCC/CCGTTGCTCACAGACCACAG
	DES		GTGAAGATGGCCCTGGATGT/TGGTTTCTCGGAAGTTGAGG
	ACTA2		GTGATCACCATCGGAAATGAA/TCATGATGCTGTTGTAGGTGGT
	SCL		CCAACAATCGAGTGAAGAGGA/CCGGCTGTTGGTGAAGATAC
	CDH5		CCTACCAGCCCAAAGTGTGT/TGTCCTTGTCTATTGCGGAGA
	PAX6		CAGCTTCACCATGGCAAATAA/ATCATAACTCCGCCCATTCA
	KRT14		CACCTCTCCTCCTCCCAGTT/ATGACCTTGGTGCGGATTT
	NCAM1		CAGATGGGAGAGGATGGAAA/CAGACGGGAGCCTGATCTCT
	TH		TGTACTGGTTCACGGTGGAGT/TCTCAGGCTCCTCAGACAGG
	GABRR2		CTGTGCCTGCCAGAGTTTCA/ACGGCCTTGACGTAGGAGA
House-Keeping Genes (qPCR)	*GAPDH*		TCCTCTGACTTCAACAGCGA/GGGTCTTACTCCTTGGAGGC
Genotyping (sequence of target region)	*TRPM4* (Exon2); Length: 618 bp (WT)/616 (heterozygous mutation)/619 (homozygous mutation)		GAGACTCCTGAGTCTGGAGCG/CCTTGGGAGCGCTGAGTGAGG
sgRNA oligonucleotide	*TRPM4* gene specific		GTCAACTATGAACGTCGTGC
Oligo for cloning sgRNA	TRPM4_guide primers		CACCGTCAACTATGAACGTCGTGC/AAACGCACGACGTTCATAGTTGACC
	U6-Fw primer for sequencing		GACTATCATATGCTTACCGT
Genomic target sequence(s)	*TRPM4* Exon 2		Homo sapiens chromosome 19, GRCh38.p13: 49157792–49211841
Top off-target mutagenesis predicted site sequencing (for CRISPR/Cas9)	Chr.22_*PI4KA*		ATGGAAAATGATAGGCCTAACTAGG /AAATGTATAAATGGGGTGGGTGC

4. GenotypingV

The genotype of the clones was determined by PCR followed by Sanger sequencing (Eurofins MWG Operon) (Primers are listed in Table 2).

4.1. Karyotyping

Karyotyping was performed at the Institute of Human Genetics of the Technical University of Munich via metaphase preparation and G-banding (≥20 metaphases counted).

4.2. Trilineage differentiation

Cells were differentiated into the three germ layers in 2D using the StemMACS™ Trilineage Differentiation Kit (Miltenyi Biotec, 130-115-660) according to manufacturer's instructions.

4.3. STR analysis

STR analysis was performed by Eurofins MWG Operon.

4.4. Off-target analysis

Potential off-target sites were predicted using CRISPOR (http://crispor.tefor.net). The top sequence was amplified (Primers are listed in Table 2) and verified by Sanger sequencing.

4.5. Differentiation towards cardiomyocytes

iPSCs were differentiated towards cardiomyocytes as described in Foo et al. (2018).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2022.102731.

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