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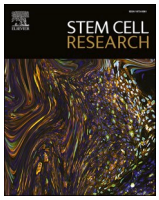

Lab Resource: Genetically-Modified Single Cell Line

Generation of an RBM20-mutation-associated left-ventricular non-compaction cardiomyopathy iPSC line (UMGi255-A) into a DCM genetic background to investigate monogenetic cardiomyopathies

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A B S T R A C T

RBM20 mutations account for 3 % of genetic cardiomypathies and manifest with high penetrance and arrhythmogenic effects. Numerous mutations in the conserved RS domain have been described as causing dilated cardiomyopathy (DCM), whereas a particular mutation (p.R634L) drives development of a different cardiac phenotype: left-ventricular non-compaction cardiomyopathy. We generated a mutation-induced pluripotent stem cell (iPSC) line in which the RBM20-LVNC mutation p.R634L was introduced into a DCM patient line with rescued RBM20-p.R634W mutation. These DCM-634L-iPSC can be differentiated into functional cardiomyocytes to test whether this *RBM20* mutation induces development of the LVNC phenotype within the genetic context of a DCM patient.

Unique stem cell line identifier	*UMGi255-A*
Alternative name(s) of stem cell line	*resDCM-634L-clone5*
Institution	*Institute of Pharmacology and Toxicology; University Würzburg*
Contact information of the reported cell line distributor	*Prof. Dr. rer. nat. Katrin Streckfuss-Bömeke: katrin.streckfuss-boemeke@uni-wuerzburg.de;* katrin.streckfuss@med.uni-goettingen.de
Type of cell line	*Human induced pluripotent stem cell (hiPSC)*
Origin	*human*
Additional origin info *(applicable for human ESC or iPSC)*	*Sex: Male* *Caucasian* *Age: 45*
Cell Source	*Blood*
Method of reprogramming	*Sendai virus*
Clonality	*Clonal*
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	*N/A*
The cell culture system used	*Feeder free culture with chemically defined medium*

(*continued on next column*)

(*continued*)

Type of the Genetic Modification	*Single nucleotide exchange with the CRISPR/Cas9 system*
Associated disease	*Gene-edited mutation is associated with left ventricle non-compaction cardiomyopathy*
Gene/locus	*RBM20 p.R634L/c.1901G > T mutation (heterozygous)* *Chromosome 10:110812298*
Method of modification/user-customisable nuclease (UCN) used, the resource used for design optimisation	*Site-specific nuclease CRISPR/Cas9 from IDT*
User-customisable nuclease (UCN) delivery method	*crRNA-Cas9 RNP complex delivered with electroporation*
All double-stranded DNA genetic material molecules introduced into the cells	*No dsDNA fragments used; HDR template as ssDNA; Cas9, gRNA1, tracrRNA*
Analysis of the nuclease-targeted allele status	*Sequencing of targeted allele by Sanger sequencing*
Method of the off-target nuclease activity prediction and surveillance	*Targeted PCR and Sanger sequencing of eight off-targets. Off target prediction by CRISPR-Cas9 guide RNA design checker tool from IDT (CRISPR-Cas9 guide RNA design checker | IDT (idtdna.com))*
Descriptive name of the transgene	*N/A*

(*continued on next page*)

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Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Light microscopy and photography	*Brightfield images show typical round stem cell-like colony growth.*	Fig. 1 *Panel C*
Pluripotency status evidence for the described cell line	Qualitative analysis *(Immunocytochemistry)*	*Positive immunostaining of pluripotency markers OCT3/4, SOX2, LIN28 and TRA1-60*	Fig. 1 *Panel C*
	Quantitative analysis *(RT-qPCR)*	*Gene-edited iPSC express pluripotency genes OCT4, SOX2 and GDF3 at similar mRNA levels as unedited and published DCM- and control-iPSC lines. iPSC-derived cardiomyocytes (NC) show low expression of corresponding genes.*	Fig. 1 *Panel D*
Karyotype	Genome-wide karyotyping via Illumina BeadArray (Infinium Global Screening Array-24 Kit)	*46,XY; genotyping of 700 000 markers*	Fig. 1 *Panel G*
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site or targeted allele-specific PCR	*Edited iPSC line possesses a heterozygous RBM20 mutation, leading to an amino acid switch from Arginine to Leucin.*	Fig. 1 *Panel B*
	Evaluation of the - (homo-/hetero-/hemi-) zygous status of introduced genomic alteration(s)	*Edited iPSC line possesses a heterozygous RBM20 mutation.*	Fig. 1 *Panel B*
	Transgene-specific PCR (when applicable)	*N/A*	*N/A*
Verification of the absence of random plasmid integration events	N/A	*N/A*	*N/A*
Parental and modified cell line genetic identity evidence	STR analysis by Eurofins	*16 independent loci (amelogenin, D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, vWA, TPOX, D18S51, D5S818, FGA) were analyzed and matched the parental line.*	*Submitted to archieve from the Journal*
Mutagenesis/genetic modification outcome analysis	Sequencing (genomic DNA PCR)	*Sequencing of the PCR band showed a RBM20 p.R634L/c.1901G > T mutation (heterozygous).*	Fig. 1 *Panel B*
	PCR-based analyses	*N/A*	*N/A*
	Southern Blot or WGS; western blotting (for knock-outs, KOs)	*N/A*	*N/A*
Off-target nuclease activity analysis	PCR across 8 (of 14) predicted top likely off-target sites (3 or more mismatches compared to on-target sequence)	*Demonstration of the lack of NHEJ-caused mutagenesis in the top predicted off-target Cas nuclease activity (PRDM11, NDUFAF6, H2BFWT, MCRS1, CHMP6, RNF130, chr16:-8695127, chr3:+72150399)*	Supplemental Figure 2
Specific pathogen-free status	Mycoplasma	*Negative Mycoplasma testing by PCR using specific primers: ACTCCTACGGGAGGCAGCAGT/TGCACCATCTGTCACTCTGTTAACCTC*	Supplemental Figure 1
Multilineage differentiation potential	Embryoid body formation	*Immunofluorescence staining of AFP, TUBB and α-SMA show differentiation of iPSC line into all three germlayers.*	Fig. 1 *Panel E*
Donor screening (OPTIONAL)	HIV, Hepatitis B, Hepatitis C	*Negative*	*N/A*
Genotype - additional histocompatibility info (OPTIONAL)	N/A	*N/A*	N/A
	N/A	*N/A*	N/A

(*continued*)

Eukaryotic selective agent resistance cassettes (including inducible, gene/cell type-specific)	*N/A*
Inducible/constitutive expression system details	*N/A*
Date archived/stock creation date	*Aug 2022*
Cell line repository/bank	*https://hpscreg.eu/search?q=UMGi255-A*
Ethical/GMO work approvals	*Ethical committee of University Medical Center Goettingen (Az −10/9/15) and Ethical committee of University Heidelberg (Ethical approval number: S-329/2012)*
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	*N/A*

1. Resource utility

Dilated cardiomyopathy (DCM) and left-ventricular non-compaction cardiomyopathy (LVNC) are both associated with *RBM20* mutations. Whether and how a monogenetic cause leads to distinct cardiomyopathies remains unclear. An induced pluripotent stem cell line from an RBM20-DCM patient, modified to incorporate the RBM20-LVNC mutation, provides a unique platform for investigating this question.

2. Resource details

DCM and LVNC are cardiac diseases that affect the myocardium. A genetic predisposition for both cardiomyopathies has been found to be correlated with the same gene: *RBM20*. Whereas the heterozygous missense mutation p.R634W leads to DCM, another amino acid substitution at the same position leads to LVNC, p.R634L (Sedaghat-Hamedani et al., 2017). These mutations are located in the conserved RS domain of *RBM20*, where 11 previously identified disease-causing mutations cluster. Cardiomyopathies resulting from *RBM20* mutations manifest aggressively within families with high penetrance, and frequently lead to cardiac arrhythmias and sudden cardiac death (Koelemen et al., 2021). The generation of patient-specific induced pluripotent stem cells (iPSC) combined with genetic modifications provides a unique opportunity to study genetic cardiomyopathies and to reveal their underlying pathomechanisms.

A description of the iPSC line derived from a DCM patient with the RBM20-p.R634W mutation and the corresponding rescue to wild-type *RBM20*, termed rescueDCM-iPSC, was previously published by our group (Rebs et al., 2020). We have now further modified the rescueDCM-iPSC by using CRISPR/Cas9 to introduce the LVNC-causing mutation p.R634L into the iPSC line with a genetic DCM background. In this new iPSC line, referred to as DCM-634L-iPSC, the original DCM-causing mutation p.R634W was converted into the LVNC-associated mutation p.R634L. RescueDCM-iPSC were electroporated with a ribonucleotide complex of crRNA-tracrRNA-Cas9 and a homology-directed repair (HDR) template (Table 2) (Panel A). Successful editing was verified by Sanger sequencing and showed a heterozygous nucleotide edit from CGG (Arg) to CTG (Leu) at position c.1901, protein position 634 (Panel B). 15 of 30 screened clones had the desired edit, resulting in an editing efficiency of 50 % for a heterozygous edit. DCM-634L-iPSC exhibited typical stem cell morphology and had high alkaline phosphatase activity (ALP) (Panel C, scale: 200 μm). Protein expression of OCT4, SOX2, LIN28, and TRA1-60 in the edited iPSC line was confirmed

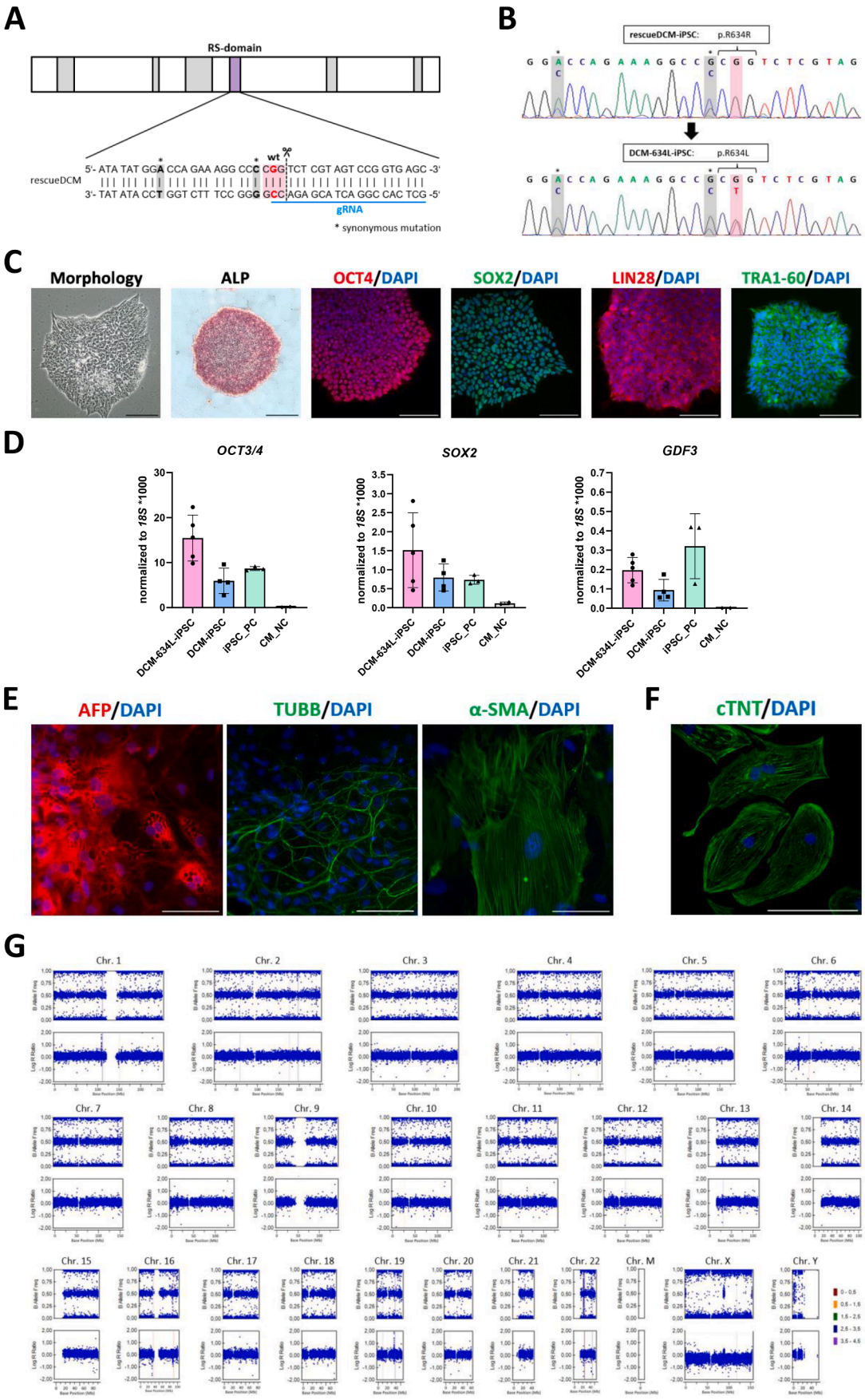

Fig. 1. Generation and characterization of DCM-634L-iPSC.

Table 2
Reagents details RRID Requirement for antibodies: use http://antibodyregistry.org/ to retrieve RRID for antibodies and include ID in the table as shown in examples.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Marker	*Mouse anti-SOX2 IgG2a*	*1:200*	*R&D, Minneapolis, Minnesota, USA, Cat# MAB2018, RRID:AB_358009*
Pluripotency Marker	*Goat anti-OCT3/4 IgG*	*1:40*	*R&D, Minneapolis, Minnesota, USA, Cat# AF1759, RRID:AB_354975*
Pluripotency Marker	*Goat anti-LIN28 IgG*	*1:300*	*R&D, Minneapolis, Minnesota, USA, Cat# AF3757, RRID:AB_2234537*
Pluripotency Marker	*Mouse anti-TRA1-60 IgM*	*1:200*	*Abcam, Cambridge, United Kingdom, Cat# ab16288, RRID:AB_778563*
Germlayer Marker	*Rabbit anti-AFP IgG*	*1:200*	*Dako, Hamburg, Germany, Cat# A0008, RRID:AB_2650473*
Germlayer Marker	*Mouse anti-α-SMA IgG2a*	*1:800*	*Sigma Aldrich, St. Louis, Missouri, USA, Cat# A2547, RRID:AB_476701*
Germlayer Marker	*Mouse anti-β-III-TubulinIgG1*	*1:1000*	*BioLegend, San Diego, California, USA, Cat# MMS-435P, RRID:AB_2313773*
Differentiation marker	*Mouse anti-cTNTIgG1*	*1:250*	*Thermo Fisher Scientific, Waltham, Massachusetts, USA, Cat# MA5-12960, RRID:AB_11000742*
Secondary antibody	*Alexa Fluor 555 donkey anti-goat IgG*	*1:1000*	*Thermo Fisher Scientific, Waltham, Massachusetts, USA, Cat# A-21432, RRID:AB_2535853*
Secondary antibody	*Alexa Fluor 488 goat anti-mouse IgG + IgM*	*1:200*	*Thermo Fisher Scientific, Waltham, Massachusetts, USA, Cat# A-10680, RRID:AB_2534062*
Secondary antibody	*Alexa Fluor 488 donkey anti-mouse IgG*	*1:1000*	*Thermo Fisher Scientific, Waltham, Massachusetts, USA, Cat# A-21202, RRID:AB_141607*
Nuclear stain	*Hoechst 33,258*	1 μg/mL	*Thermo Fisher Scientific, Waltham, Massachusetts, USA, Cat#* H3569, *RRID:AB_2651133*
Site-specific nuclease			
Nuclease information	*S.p. Cas9 Nuclease V3*	*Integrated DNA Technologies (IDT) Cat# 1,081,058*	
Delivery method	*Electroporation*	*Lonza 4D-Nucleofector; program CA-137*	
Selection/enrichment strategy	*Selection by hand/picking colonies under microscope*	*Microscope Leica Type 090–135.001*	
Primers and Oligonucleotides used in this study			
	Target	**Forward/Reverse primer (5′-3′)**	
Pluripotency Markers (qPCR)	*OCT3/4*	*CCCCAGGGCCCCATTTTGGTACC/ACCTCAGTTTGAATGCATGGGAGAGC*	
Pluripotency Markers (qPCR)	*SOX2*	*GCTACAGCATGATGCAGGACCA/TCTGCGAGCTGGTCATGGAGTT*	
Pluripotency Markers (qPCR)	*GDF3*	*GTCTCCCGAGACTTATGCTACG/AGTAGAGGAGCTTCTGCAGGCA*	
House-Keeping Genes (qPCR)	*18S*	*ACCCGTTGAACCCCATTCGTGA/GCCTCACTAAACCATCCAATCGG*	
Targeted mutation analysis/sequencing	*RBM20*	*GAGTGTACACAGTTACATGCAC/GTGGGACCTCGGGGAGA*	
Potential random integration-detecting PCRs		*N/A*	
crRNA sequence	*RBM20 Exon9*	*GCTCACCGGACTACGAGACC (targets - strand)*	
Genomic target sequence(s)	*PAM CGG*	*GGTCTCGTAGTCCGGTGAGCCGGG (PAM) (+strand)*	
Bioinformatic gRNA on– and -off-target binding prediction tool used, specific sequence/outputs link(s)	*IDT*	*Off target prediction by CRISPR-Cas9 guide RNA design checker tool from IDT (CRISPR-Cas9 guide RNA design checker | IDT (www.idtdna.com)*	
Primers for top off-target mutagenesis predicted site sequencing (for all CRISPR/Cas9, ZFN and TALENs)	*OT1 – PRDM11*	*CAGGGAGGGTAACCAGACTC/GAGAACGTTTCCCAGGGTTC*	
	OT2 – NDUFAF6	*GCCCCAGATAGAGGGGTAGA/AACAGAGGGGCTAAGTTCCG*	
	OT3 – H2BFWT	*GAGTAGTGCATTTGGGCAGC/CTCCGGACATGTAGGGTCAC*	
	OT4- MCRS1	*CTGGTTCCCTGCCTTGCAT/AGGCCAGAGAAAGATGGGAAAC*	
	OT5 – CHMP6	*AAAGGACCTCCGATGCTTGG/CTCCCTTTGTCCTTGTGGCT*	
	OT6 – RNF130	*CCTAATAACCGCTGCTGGTG/TACTAGCTGCTATGCTGGGG*	
	OT7 – chr16:-8695127 (non-coding)	*CCCCATTCCCGACGTTTCAC/TTCTCCCAAAACCGCGTAGC*	
	OT8 – chr3:+72150399 (non-coding)	*CCGGTTTCCTCCCACTTCAC/TCCACAGTCATGCGCCATTT*	
ODNs/plasmids/RNA molecules used as templates for HDR-mediated site-directed mutagenesis	*ssDNA*	*IDT-HDR -template*	
		*G*G*TGTGAAGATTCTAAATCCTGCTCCTTGGCTCCCTCACAGATATGGACCAGAAAGGCCGCTGTCTCGTAGTCCCGTGAGCCGGTCACTCTCCCC*G*A*	

by immunofluorescence staining (Panel C, scale: 100 μm). In addition, the cells exhibited mRNA levels of the pluripotency genes *OCT3/4, SOX2* and *GDF3* that were comparable to those in their unedited parental DCM-iPSC line (Rebs et al., 2020) and a second published control iPSC line (Borchert et al., 2017), while differentiated iPSC-derived cardiomyocytes served as a negative control (Panel D). To demonstrate the cells' differentiation capacity, an *in vitro* trilineage differentiation was performed via embryoid body formation in coculture with mouse embryonic fibroblasts (MEF). Positive expression of endodermal α-fetoprotein (AFP), ectodermal β-III-Tubulin (TUBB), and mesodermal α-smooth muscle actin (α-SMA) were verified by immunofluorescence staining (Panel E).

As *RBM20* mutations affect the structure and the function of the cardiac muscle, our primary focus lies in the investigation of cardiomyocytes. Consequently, the DCM-634L-iPSC were successfully differentiated into beating cardiomyocytes that were positive for cardiac troponin T (cTNT) (Panel F).

The cell line maintains a normal 46,XY karyotype (Panel G), and the absence of mycoplasma contamination was routinely validated every two weeks by PCR and subsequent gel electrophoresis (Supp. Fig. 1). An STR analysis confirmed that the loci matched the patient line and corresponding rescue line.

3. Materials and methods

3.1. Gene editing

2×10^6 iPSC were electroporated with a CRISPR ribonucleoprotein (CRISPR-RNP) complex consisting of 5 μl crRNA (100 μM stock), 5 μl tracrRNA (100 μM stock), 2 μl Cas9 protein (10 μg/ml stock), 1 μl electroporation enhancer, and 3 μg HDR template (Integrated DNA Technologies). For electroporation, 2×10^6 iPSC were counted and centrifuged at 200 g for 5 min. Electroporation solution was prepared by mixing 82 μl nucleofection solution and 18 μl nucleofection supplement (P3 Primary Cell 4D-Nucleofector X Kit L, Lonza) with the CRISPR-RNP complex. The iPSC pellet was dissolved in electroporation solution, transferred to an electroporation cuvette, and nucleofected using a 4D-Nucleofector (program CA-137; Lonza). Cells were transferred to a 6-well plate containing StemFlex medium supplemented with 2 μM Thiazovivin (Merck). On day (d) 3, iPSC (800–1600 cells per 6-well plate) were plated to produce single-cell-based colonies. On d7 to d10, suitable colonies were picked and cultivated. For screening, gDNA was isolated (DNA Mini Kit, Qiagen), the *RBM20* exon 9 locus was amplified by PCR, and the PCR product was sequenced by Sanger sequencing (Microsynth).

3.2. Cell culture and cardiac differentiation

iPSC were maintained in StemFlex medium (refreshed every other day; Thermo Fisher Scientific). Cells were passaged at 80–90 % confluency, as previously described (Borchert et al., 2017). Differentiation into ventricular cardiomyocytes was performed via manipulation of the Wnt signaling pathway (Streckfuss-Bömeke et al., 2017).

3.3. Genotyping and sequence analysis

DNA was isolated from iPSC clones using QIAamp DNA mini kits (Thermo Fisher Scientific). The *RBM20* exon 9 locus was amplified by PCR with specific primers (Table 2); the product was Sanger sequenced by Microsynth Seqlab (Göttingen, Germany).

3.4. Spontaneous in vitro differentiation

Spontaneous *in vitro* differentiation was performed by coculturing iPSC with inactivated MEF in embryoid bodies. 5.3×10^6 iPSC were mixed with 2.6×10^6 MEF in 20 ml StemFlex medium, centrifuged at 250 g for 5 min, and distributed into a U-bottom 96-well plate (200 μl per well). On d1, medium was replaced with IMDM medium (Thermo Fisher Scientific) supplemented with 450 μM monothioglycerol, 20 % FCS and 1 × NEAA. Embryoid bodies were replated into Geltrex-coated dishes on d8 and analyzed on d8 + 8 and d8 + 25.

3.5. Mycoplasma detection

Mycoplasma screening was routinely performed using specific primers (see Table 1) in a semi-quantitative PCR, followed by 1.5 % agarose gel electrophoresis.

3.6. RNA isolation, cDNA synthesis, and qPCR

RNA was isolated using the ReliaPrep RNA Tissue Miniprep kit (Promega) per manufacturer instructions for "Purification of RNA from Fibrous Tissues". 200 ng RNA were reverse-transcribed using the iScript cDNA Synthesis kit (Bio-Rad) per manufacturer instructions. Quantitative PCR was performed with 300 ng RNA, pluripotency-gene-specific primers (500 nM) and 1 × SYBR Green (Bio-Rad) using the CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Gene expression was normalized to *18S*.

3.7. Immunofluorescence

Cells were fixated with Histofix (Carl Roth) for 20 min at RT and blocked in 1 % BSA/PBS for 1 h at RT. Antibodies were diluted in 0.1 % Triton in 1 % BSA/PBS (Table 2). Primary antibodies were incubated overnight at 4 °C; fluorescently labelled secondary antibodies were applied in combination with Hoechst 33,258 for 1 h at 37 °C. Images were acquired with a DMi8 fluorescence microscope and LasX software (Leica).

3.8. STR analysis

Genomic DNA was isolated using the QIAamp DNA mini kit (Qiagen). STR analysis was performed by Eurofins Germany. 16 gene loci were analyzed: D21S11, D7S820, D8S1179, D3S1358, CSF1PO, TH01, D13S317, D16S539, D19S433, D2S1338, AMEL, D5S818, FGA, vWA, TPOX, and D18S51.

3.9. Karyotyping

Molecular karyotyping was performed at Life & Brain (Bonn, Germany) via genome-wide array-based genotyping using the Illumina BeadArray. Data were analyzed in GenomeStudio v2.0 (Illumina) with the cnvPartition 3.2.0 plug-in.

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.

Acknowledgments

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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.2023.103290.

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