Stem Cell Research 53 (2021) 102256

Contents lists available at ScienceDirect

Stem Cell Research

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

Lab Resource: Multiple Cell Lines

CRISPR/Cas9-edited PKP2 knock-out (JMUi001-A-2) and DSG2 knock-out (JMUi001-A-3) iPSC lines as an isogenic human model system for arrhythmogenic cardiomyopathy (ACM)

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A B S T R A C T

Arrhythmogenic cardiomyopathy (ACM) is characterized by fibro-fatty replacement of the myocardium, heart failure and life-threatening ventricular arrhythmias. Causal mutations were identified in genes encoding for proteins of the desmosomes, predominantly plakophilin-2 (PKP2) and desmoglein-2 (DSG2). We generated gene-edited knock-out iPSC lines for PKP2 (JMUi001-A-2) and DSG2 (JMUi001-A-3) using the CRISPR/Cas9 system in a healthy control iPSC background (JMUi001-A). Stem cell-like morphology, robust expression of pluripotency markers, embryoid body formation and normal karyotypes confirmed the generation of high quality iPSCs to provide a novel isogenic human *in vitro* model system mimicking ACM when differentiated into cardiomyocytes.

1. Resource table

Unique stem cell lines identifier	JMUi001-A-2 JMUi001-A-3
Alternative names of stem cell lines	PKP2-KO (JMUi001-A-2) DSG2-KO (JMUi001-A-3)
Institution	Comprehensive Heart Failure Center (CHFC), Department of Cardiovascular Genetics, University Clinics Würzburg, Würzburg, Germany
Contact information of distributor	Brenda Gerull, gerull_b@ukw.de
Type of cell lines	iPSC
Origin	Human
Cell Source	iPSC
Clonality	Clonal
Method of reprogramming	JMUi001-A via lentivirus
Multiline rationale	Genome-edited disease cell lines
Gene modification	Yes
Type of modification	Homozygous induced mutation
Associated disease	Arrhythmogenic cardiomyopathy (ACM)
Gene/locus	NM_001005242 (*PKP2*):c.[142_145delGGCC;148C>A];[142_145delGGCC;148C>A] NM_001943.5 (*DSG2*):c.[9_24del16];[9_24del16]
Method of modification	CRISPR/Cas9
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	original dermal fibroblasts of JMUi001-A were obtained by a commercial provider (PromoCell, #C-12300)

2. Resource utility

Phenotypic variabilities impede the identification of ACM-associated pathomechanisms. Therefore, we aim to provide a powerful tool for research through the generation of an isogenic model system of ACM using CRISPR/Cas9 technology in healthy human iPSCs to generate

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https://doi.org/10.1016/j.scr.2021.102256
Received 26 January 2021; Received in revised form 26 January 2021; Accepted 13 February 2021
Available online 18 February 2021

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
JMUi001-A-2	PKP2-KO	Male	6	Caucasian	NM_001005242 (*PKP2*): c.[142_145delGGCC;148C>A];[142_145delGGCC;148C>A]	Arrhythmogenic cardiomyopathy (ACM)
JMUi001-A-3	DSG2-KO	Male	6	Caucasian	NM_001943.5 (*DSG2*): c.[9_24del16];[9_24del16]	Arrhythmogenic cardiomyopathy (ACM)

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Typical embryonic stem cell-like morphology	Fig. 1 panel C
Phenotype	Qualitative analysis	Positive expression of pluripotency markers OCT3/4, SOX2 and TRA-1–81 via immunofluorescence.	Fig. 1 panel C
	Quantitative analysis (Flow cytometry)	Expression of SSEA-4 and TRA-1–60 in high purities in all generated cell lines. PKP2-KO: 98.2% TRA-1–60$^+$, 92.1% SSEA-4$^+$ DSG2-KO: 92.3% TRA-1–60$^+$, 97.6% SSEA-4$^+$	Fig. 1 panel D
Genotype	Karyotype (G-banding) and resolution	PKP2-KO: 46,XY DSG2-KO: 46,XY Resolution: 450	Fig. 1 panel B
Identity	Microsatellite PCR (mPCR) OR STR analysis	N/A STR analysis of: AMEL, D3S1358, SE33, TH01, D18S51, FGA, vWA, D21S11, D8S1179. Both generated iPSC lines match to the individual host profile.	N/A submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	PKP2-KO: NM_001005242 (*PKP2*):c.[142_145delGGCC;148C>A];[142_145delGGCC;148C>A] DSG2-KO: NM_001943.5 (*DSG2*): c.[9_24del16];[142_145delGGCC;148C>A]	Fig. 1 panel A
	Southern Blot OR WGS	The most likely off-targets (gRNA mismatches) CETN1 (NM_004066), NTN3 (NM_006181) and AGPAT2 (NM_001012727) for PKP2-KO and JAKMIP1 (NM_144720), SLC35B4 (NM_032826) and CAPZB (NM_004930) for DSG2-KO were not changed after CRISPR/Cas9 modification.	Supplementary Fig. 1
Microbiology and virology	Mycoplasma	Mycoplasma screening was performed by Eurofins Genomics. Both iPSC lines were mycoplasma negative.	submitted in archive with journal
Differentiation potential	Embryoid body formation	Both iPSC lines were differentiated into cells of mesoderm, endoderm and ectoderm by expression of α-smooth muscle actin (α-SMA), α-feto protein (AFP), tubulin-β-III (TUBB3).	Fig. 1 panel F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping HLA tissue typing	N/A N/A	N/A N/A

homozygous *PKP2* knock-out and *DSG2* knock-out iPSC lines.

3. Resource details

Clinical features of arrhythmogenic cardiomyopathy (ACM) are heart failure due to dysfunction of the right and/or left ventricle, fibro-fatty infiltration of the myocardium and life-threatening ventricular arrhythmias. ACM is caused by autosomal dominant and recessive mutations in genes encoding desmosomal proteins like plakophilin-2 (PKP2, MIM #602861) (Gerull et al., 2004) and desmoglein-2 (DSG2, MIM #125671) (Pilichou et al., 2006). Desmosomes are part of adherens junctions and located in the intercalated discs. They maintain tissue integrity, whereas functional knock-outs of desmosomal proteins negatively affect cell-to-cell adhesion. The detailed pathomechanisms of how desmosomal dysfunction leads to the disease remains elusive. However, it has been hypothesized that a transcriptional switch from myogenesis to adipogenesis and fibrogenesis might be causative for the fibro-fatty myocardial degeneration (Austin et al., 2019). To date, high variations among clinical phenotypes of mutation carriers make it difficult to use reprogrammed patient-derived iPSC lines only. Therefore, we aim to provide an isogenic system by generating human PKP2 and DSG2 knock-out iPSCs in the genetic background of healthy control iPSCs (see Tables 1 and 2).

The CRISPR/Cas9 system was implemented in control iPSCs (JMUi001-A, (Kwok et al., 2018)) by using self-designed sgRNAs homologous to *PKP2* exon 1 and *DSG2* exon 1. After nucleofection of both systems, single cell colonies were expanded and analysed for genetic alterations. One representative CRISPR clone was selected for PKP2-KO that carries the homozygous mutation NM_001005242 (*PKP2*):c.[142_145delGGCC;148C>A];[142_145delGGCC;148C>A] and one for DSG2-KO that carries the homozygous mutation NM_001943.5 *(DSG2)*: c.[9_24del16];[9_24del16] (Fig. 1A). We observed no numerical or structural chromosomal alterations after performing conventional karyotype analysis in PKP2-KO and DSG2-KO iPSCs (Fig. 1B). Bright field images confirmed a typical stem cell-like morphology (Fig. 1C). Key pluripotency markers were investigated via immunofluorescence and revealed robust nuclear expression of OCT3/4 and SOX2 as well as the expression of the stem cell glycoprotein TRA-1–81 (Fig. 1C). Flow cytometry was performed and unravelled a strong expression of stem cell surface markers accompanied by high iPSC population purities with 92.1% SSEA-4$^+$ and 98.2% TRA-1-61$^+$ for PKP2-KO iPSCs and 97.6% SSEA-4$^+$ and 92.3% TRA-1-61$^+$ for DSG2-KO iPSCs (Fig. 1D). In both iPSC lines a frameshift and a premature termination codon is predicted to cause loss of PKP2 or DSG2, respectively, which was confirmed by western blot (Fig. 1E) and immunofluorescence analysis (Supplementary Fig. 1). Moreover, non-directed germ layer differentiation was addressed to prove pluripotent capacities. In accordance progenies of both iPSC lines showed robust expression for mesodermal proteins like α-SMA, ectodermal fate represented by TUBB3 and endoderm formation via AFP after immunofluorescence staining (Fig. 1F). The genetic identity of PKP2-KO and DSG-KO iPSCs with the healthy control iPSC line (JMUi001-A) was confirmed via STR analysis and Sanger sequencing of off-targets (Supplementary Fig. 1). Both KO iPSCs were mycoplasma negative that proved the high quality of the iPSCs.

Both generated iPSC lines showed typical pluripotency hallmarks by expressing key pluripotency proteins, germ layer differentiation potential and normal karyotypes. Therefore, we provide a novel isogenic model system to study ACM in genetically modified iPSCs, thus,

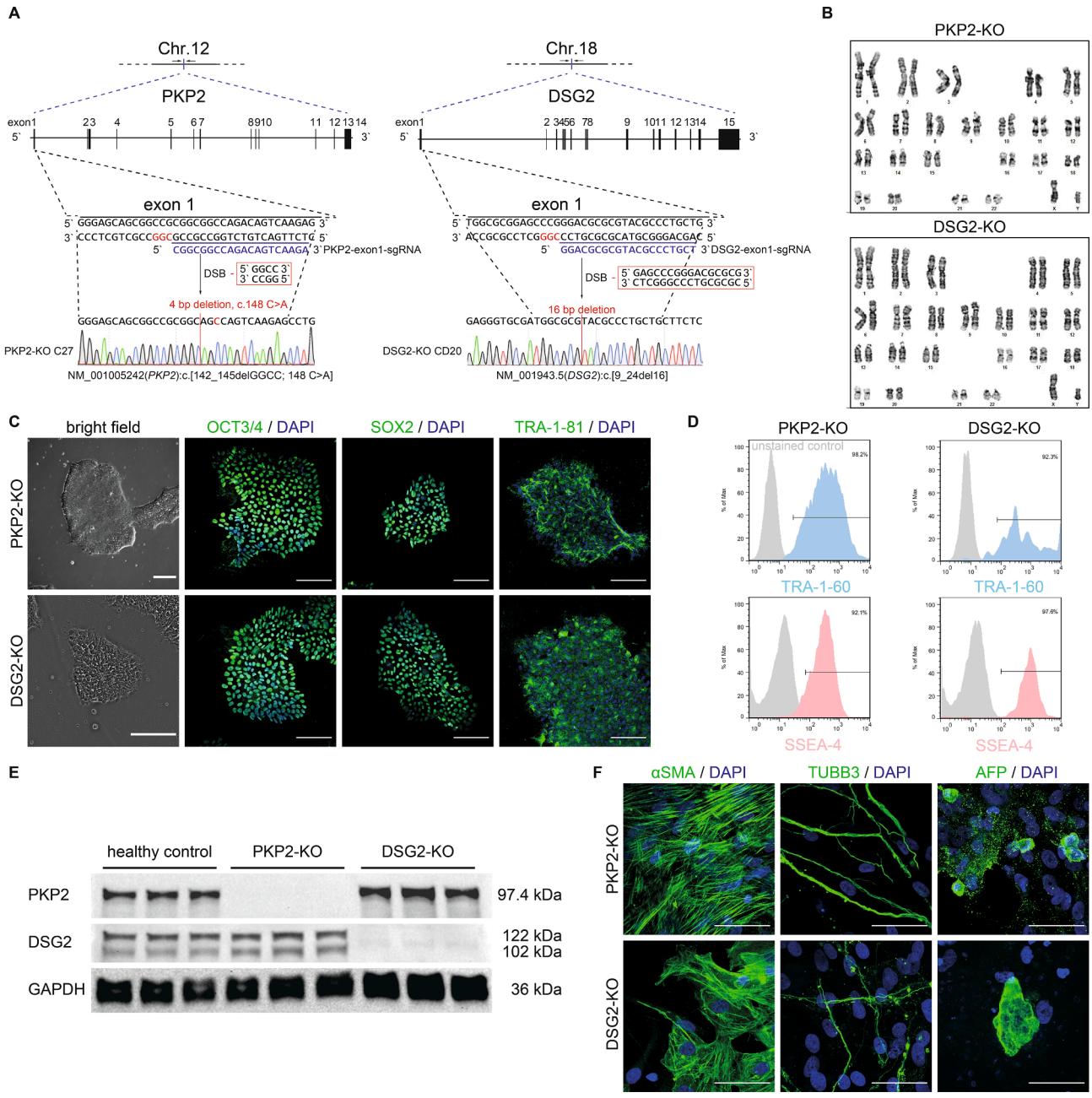

Fig. 1. Generation and characterization of PKP2-KO and DSG2-KO iPSC lines using CRISPR/Cas9 technology.

allowing the identification of novel pathomechanisms and potentially to test novel therapeutics.

4. Materials and methods

4.1. Cultivation of iPSCs

iPSCs were cultured on Matrigel™ (Corning) coated wells in mTeSR™1 (STEMCELL) with daily medium change. Accutase (Gibco) and mTeSR™1 supplemented with 10 μM Y-27632 (Miltenyi) were used for passaging.

4.2. CRISPR/Cas9

The CRISPR tool (http://tools.genome-engineering.org) was used to design PKP2-exon1-sgRNA and DSG2-exon1-sgRNA (Table 3) that were cloned into the pSpCas9(BB)-2A-Puro(PX459) plasmid (Addgene) (Ran et al., 2013). Gene sequences with the highest probability to be targeted and the lowest number of mismatches were chosen. 250,000 healthy iPSCs (JMUi001-A, (Kwok et al., 2018)) were nucleofected with 35 μg plasmid using the NEON™ Transfection System (Invitrogen) with adjusted parameters [Voltage: 1200 V, Pulse length: 35 ms, Number of pulses: 2]. iPSCs were reseeded into coated 10 cm dishes with mTeSR™1 containing 10 μM Y-27632. One day after, medium was changed into mTeSR™1 supplemented with 1 μg/ml Puromycin (Life Technologies) for 24 h. After ~3 weeks single-cell-derived surviving colonies were manually picked and individually transferred into pre-coated wells of a 96-well plate (Ran et al., 2013). After clonal expansion, iPSCs were collected for cryopreservation and DNA isolation.

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency marker	Mouse anti-OCT3/4 (Octamer-binding transcription factor-3/4) IgG_{2B}	1:50	Santa Cruz Biotechnology, Cat#sc-5279, RRID: AB_628051
Pluripotency marker	Mouse anti-SOX2 (sex determining region (SRY)-box 2) IgG_{2A}	1:50	R&D, Cat#MAB2018, RRID: AB_358009
Pluripotency marker	Mouse anti-TRA-1–81 IgM	1:50	STEMCELL Technologies, Cat#60065, RRID: AB_2721032
Endoderm marker	Rabbit anti-AFP (α-1-fetoprotein) IgG	1:500	Agilent, Cat#A0008, RRID: AB_2650473
Ectoderm marker	Mouse anti-TUBB3 (tubulinβ-III) IgG_{2A}	1:1000	BioLegend, Cat#801201, RRID: AB_2313773
Mesoderm marker	Rabbit anti-α-SMA (α-smooth muscle actin) IgG	1:100	Abcam, Cat#ab5694, RRID: AB_2223021
PKP2-mutation analysis	Mouse anti-PKP2 igG_1	1:2.5	Progen, Cat#651101, RRID: AB_1542758
DSG2-mutation analysis	Mouse anti-DSG1/2 igG_1	1:10	Progen, Cat#61002, RRID: AB_1541105
GAPDH	Rabbit anti-GAPDH (HRP conjugate) IgG	1:2000	Cell Signaling Technology, Cat#3683S, RRID: AB_1642205
Secondary antibody	Alexa Fluor 488, Donkey anti-Mouse IgG	1:1000	Thermo Fisher Scientific, Cat#A21202, RRID: AB_141607
Secondary antibody	Alexa Fluor 594, Donkey anti-Rabbit IgG	1:1000	Thermo Fisher Scientific, Cat#A21207, RRID: AB_141637
Flow cytometry	Human anti-TRA-1–60-PE	1:11	Miltenyi Biotec, Cat#130-100-347, RRID: AB_2654227
Flow cytometry	Human anti-SSEA-4-APC	1:11	Miltenyi Biotec, Cat#130-098-347, RRID: AB_2653520
Primers			
	Target	Forward/Reverse primer (5′–3′)	
PKP2-exon1-sgRNA	PKP2 exon1	GTCTTGACTGTCTGGCCGCCG/ CGGCGGCCAGACAGTCAAGA	
DSG2-exon1-sgRNA	DSG2 exon1	AGCAGGGCGTACGCGCGTCC/ GGACGCGCGTACGCCCTGCT	
PKP2 mutation sequencing	PKP2 exon1	CCAGCTGAGTACGGCTACAT/ TTTAGGAACAGGGGAACGGC	
DSG2 mutation sequencing	DSG2 exon1	CCCGGCTCCATTTTCTCGC/ CTAGACCTCGCCTACCGACG	
PKP2-KO: Off-target sequencing 1	CETN1 (NM_004066), 4MMS (3:6:8:9)	GACAGCGGATTCTCGCCTAA/ CCGATCAGCTTCGTCGATCA	
PKP2-KO: Off-target sequencing 2	NTN3 (NM_006181), 4MMS (2:3:4:12)	CTTCTACTGCGACAGGCCAT/ GTGGTCTGGTTGCAGGTCTT	
PKP2-KO: Off-target sequencing 3	AGPAT2 (NM_001012727), 4MMS (4:5:8:12)	CCGAGTTCTACGCCAAGGT/ AGGGAAGCCCAGAAGAAAGTT	
DSG2-KO: Off-target sequencing 1	JAKMIP1 (NM_144720), 3MMS (12:19:20)	AGCACTGTGAGATGTGGTCG/ GACCTTGCTATGGGTTCCCC	
DSG2-KO: Off-target sequencing 1	SLC35B4 (NM_032826), 4MMS (1:2:11:12)	GCGAACTACAGCTTCTTGGC/ CTGCCCAGACAGGACGGATT	
DSG2-KO: Off-target sequencing 1	CAPZB (NM_004930), 3MMS (4:9:10:13)	CAGTTAGCGCTGGCCTTCTG/ CACGTGGCACTAGGAACTCA	

4.3. Genotyping and sequencing

DNA was isolated (DNeasy Kit; Qiagen) and corresponding off-targets of *PKP2* and *DSG2* were amplified by PCR (Table 3). PCR products were purified (PCR Purification Kit; Qiagen) and sequenced (Microsynth). Genetic alterations were identified using SnapGene3.3.3.

4.4. STR analysis

Individual STR profiles were examined on eight independent genomic loci with the Mentype®Nonaplex I PCR Amplification Kit (Biotype) using 2 ng DNA.

4.5. Western blot

Cell pellets were lysed and 50 μg protein each was loaded on a 12% polyacrylamide gel. Proteins were transferred on a nitrocellulose membrane, blocked and stained with primary antibodies (Table 3). After secondary antibody incubation chemiluminescence was visualized with the ChemiDoc system (BIO-RAD).

4.6. Immunofluorescence

iPSCs were seeded on pre-coated coverslips and fixed with 4% paraformaldehyde (Sigma-Aldrich). iPSCs were permeabilized with 0.2% Nonidet-P40 (US Biological), blocked with 10% donkey serum (Sigma-Aldrich) and incubated with primary antibodies (Table 3) overnight at 4 °C. Secondary antibodies were incubated for 2 h at RT followed by DAPI (Roth) staining. Images were acquired with the LSM780 (Zeiss) and processed using ImageJ.

4.7. Flow cytometry

iPSCs were stained with FACS antibodies (Table 3). Positively labeled iPSCs were used for gating by using BD FACSCalibur with the appropriate laser and filter sets. FlowJo7.6.1 software was used to quantify population purities.

4.8. Karyotype

iPSCs were treated with Colcemid™ [10 μg/ml] and analysis was performed on 10 GTG-banded metaphases. Images were acquired with a resolution of 300–500 bands per haploid karyotype using an Axioskop (Zeiss) microscope.

4.9. Non-directed differentiation

A single cell suspension of 10 million iPSCs was seeded in non-coated non-TC-treated dishes in mTeSR™1 containing + 10 μM Y-27632. mTeSR™1 was changed daily until aggregates formed. After one week, medium was changed to differentiation medium [DMEM (Gibco), 10% FCS (Sigma-Aldrich), 1% MEM-NEAA (Gibco), 100 μM β-mercaptoethanol (Thermo)]. One week later, ~10 aggregates were placed on 0.1% gelatin coated coverslips in differentiation medium. Medium was changed every 3 days to allow default germ layer formation that was assessed after 8 weeks via immunofluorescence.

Mycoplasma analysis

iPSC cultured medium was collected individually and tested for mycoplasma contamination (Eurofins).

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

The authors thank Anne Kilinç, Martina Regensburger, Noemi Knötgen and Birgit Halliger-Keller for their excellent technical assistance and their support. This work was supported by the Federal Ministry of Education and Research (BMBF)[grant number 01EO1504].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2021.102256.

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