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Lab Resource: Genetically-Modified Multiple Cell Lines

Generation of human induced pluripotent stem cell line EURACi006-A and its isogenic gene-corrected line EURACi006-A-1 from an arrhythmogenic cardiomyopathy patient carrying the c.1643delG *PKP2* mutation

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A B S T R A C T

Arrhythmogenic Cardiomyopathy (ACM) is a rare genetic cardiac disease predominantly associated with mutations in genes of the desmosomes and characterized by arrhythmia and fibro-fatty replacement of the myocardium. We generated human induced pluripotent stem cells (hiPSCs) from one patient affected by ACM carrying the heterozygous c.1643delG (p.G548VfsX15) *PKP2* mutation and then corrected the mutation using CRISPR/Cas9 technology. Both original and corrected hiPSC lines showed typical morphology of pluripotent cells, expressed pluripotency markers, displayed a normal karyotype, and differentiated towards the three germ layers. This isogenic hiPSC pair can be used to study the role of the c.1643delG *PKP2* mutation *in vitro*.

Resource Table:

Unique stem cell lines identifier	1. EURACi006-A 2. EURACi006-A-1
Alternative name(s) of stem cell lines	1. M003#9.3 2. iso M003#9.3-C12/LUMCi047-A-1
Institution	Institute for Biomedicine, Eurac Research Leiden University Medical Center, LUMC
Contact information of the reported cell line distributor	Alessandra Rossini (alessandra.rossini@eurac.edu) (EURACi006-A) Milena Bellin (m.bellin@lumc.nl) (EURACi006-A-1) Viviana Meraviglia (v.meraviglia@lumc.nl) (EURACi006-A-1)
Type of cell lines	iPSCs
Origin	Human
Additional origin info *(applicable for human ESC or iPSC)*	Age: 44 Sex: Male Ethnicity: Caucasian
Cell Source	Skin fibroblasts
Method of reprogramming	Non-integrating episomal vectors (pCXLE hOCT3/4-shp53-F, pCXLE-hSK, and pCXLE-hUL)
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	qPCR to exclude the presence of episomal plasmids
Cell culture system used	Cells grown on MEFs during reprogramming and then feeder-free condition using Essential 8™ Medium
Type of Genetic Modification	Spontaneous pathogenic mutation (EURACi006-A) Gene correction of pathogenic mutation (EURACi006-A-1)
Associated disease	Arrhythmogenic Cardiomyopathy (OMIM: 609040)
Gene/locus	Heterozygous *PKP2* c.1643delG/ 12p11.21 (dbSNP: rs794729137)
Method of modification/site-specific nuclease used	CRISPR/Cas9
Site-specific nuclease (SSN) delivery method	RNP electroporation
All genetic material introduced into the cells	Guide RNA (gRNA) and single-stranded oligonucleotide (ssODN) as homology directed repair (HDR) template
Analysis of the nuclease-targeted allele status	Targeted PCR/sequencing

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(*continued*)

Method of the off-target nuclease activity surveillance	Targeted PCR/sequencing
Name of transgene	N/A
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	N/A
Inducible/constitutive system details	N/A
Date archived/stock date	May 2019 (EURACi006-A) February 2020 (EURACi006-A-1)
Cell line repository/bank	EURACi006-A https://hpscreg.eu/cell-line/EURACi006-A EURACi006-A-1 https://hpscreg.eu/cell-line/EURACi006-A-1
Ethical/GMO work approvals	Skin fibroblasts were collected after patient signed informed consent and the protocol was approved by Centro Cardiologico Monzino - IRCCS Ethical Committee (12/06/2012). The generation of iPSCs was also approved by Ethical Committee of the Province of South Tyrol (Nr.1/2014, 12/03/2014) and by the Leiden University Medical Centre ethics committee under the umbrella protocol P 13.080 "Parapluprotocol: hiPSC"
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	Plasmids used for reprogramming: pCXLE-hOCT3/4-shp53-F Addgene #27077 pCXLE-hSK Addgene #27078 pCXLE-hUL Addgene #27080 pCXLE-EGFP Addgene #27082

1. Resource utility

The isogenic EURACi006-A (*PKP2*-mutated) and EURACi006-A-1 (gene-corrected) hiPSC pair can be used as unlimited source for human cardiac cells to investigate *in vitro* the molecular mechanisms of ACM pathogenesis linked to this specific *PKP2* mutation. These lines are also valuable tools to screen for drugs that might provide treatment.

2. Resource details

Arrhythmogenic cardiomyopathy (ACM, OMIM: 609040) is a rare inherited disease characterised by life-threatening arrhythmia and progressive fibro-fatty substitution of the myocardium, contributing to cardiac dysfunction and heart failure. ACM is often caused by pathogenic variants in genes encoding proteins of the desmosomes, with *PKP2* being the most common causal gene (Fressart et al. 2010). Although ACM genetic basis have been thoroughly studied and the disease diagnosis has advanced, ACM is a complex disease in which the cardiac cell type(s) responsible for fibro-fatty replacement and the pathogenic molecular mechanisms are still a matter of debate. In order to elucidate the role of patient-specific *PKP2* mutations in ACM pathophysiology, large quantities of adult primary cardiac cells are a major requirement. Given the restricted availability of cardiac biopsies from ACM patients, hiPSCs provide a solution for unlimited source of cardiac cells carrying the patient-specific mutation and genetic background. We generated the EURACi006-A line from a 44 year old male carrying the heterozygous pathogenic mutation in *PKP2* c.1643delG (NM_004572.3, dbSNP: rs794729137) that leads to a shift in the reading frame (p.G548VfsX15, NP_004563.2), creating a premature stop codon on the PKP2 protein (Perrin et al. 2013). Several hiPSC clones were successfully generated by reprogramming of skin fibroblasts using electroporation of episomal plasmids carrying *OCT3/4*, *SOX2*, *KLF4*, and *L-MYC* (Meraviglia et al. 2016) and the EURACi006-A was selected based on morphology and characterized as reported in Table 1. The EURACi006-A-1 isogenic line with the heterozygous *PKP2* c.1643delG correction was generated using CRISPR/Cas9-mediated genome editing and further characterized (Table 1). For correcting the mutation, we used CRISPR/Cas9-induced homology directed repair (HDR) and a single-stranded oligonucleotide (ssODN) as a repair template (Fig. 1A). To avoid re-cutting after HDR, two silent mutations (blue arrows, Fig. 1A) were introduced in the ssODN. The presence of *PKP2* c.1643delG in EURACi006-A and the correction of the heterozygous pathogenic mutation in the corrected line EURACi006-A-1 were confirmed by DNA sequencing (Fig. 1B). Both EURACi006-A and EURACi006-A-1 lines showed the typical human embryonic-stem cell-like morphology with high nuclear/cytoplasmatic ratio (Fig. 1C). Immunofluorescence analysis demonstrated the expression of typical pluripotency markers OCT3/4, NANOG (nuclear proteins) and SSEA4 (membrane protein) (Fig. 1D). A high percentage (>90%) of cells positive for OCT3/4, NANOG and SSEA4 was confirmed by flow cytometry analysis in both hiPSC lines (Fig. 1E). qPCR analysis excluded the integration of the plasmids used for the reprogramming in both hiPSC lines, indicating the successful activation of endogenous pluripotent genes (Supplementary Fig. 1A). Immunostaining analysis showed the ability of both lines to differentiate into the three germ lineages as indicated by positive staining for ectodermal- (NES, PAX6), mesodermal- (VIM, CDX2, T) and endodermal- (FOXA2, GATA4, EOMES) markers (Fig. 1F). Short tandem repeat (STR) indicated matching genetic identities (100% match) of EURACi006-A and EURACi006-A-1 and their parental skin fibroblasts (Table 1, available with the authors). G-banding cytogenetic analysis was performed on EURACi006-A (at passage 29) and EURACi006-A-1 (at passage 38), showing a normal karyotype (Supplementary Fig. 1B). After gene correction, off-target analysis by Sanger sequencing demonstrated lack of indels in the *in silico* predicted top-5 sites and in the coding regions of EURACi006-A-1 (Supplementary Fig. 1C). hiPSC lines were mycoplasma-free (Supplementary Fig. 1D).

3. Materials and methods

3.1. Ethical statementt

Skin fibroblasts were collected after patient written informed consent. This study was approved by the Centro Cardiologico Monzino - IRCCS Ethical Committee (12/06/2012), the Ethical Committee of the Province of South Tyrol (Nr.1/2014, 12/03/2014) and the Medical Ethical Committee at the Leiden University Medical Center (P13.080).

3.2. Cell culture and reprogramming

Skin fibroblasts were isolated from skin biopsy and cultured as previously described (Meraviglia et al. 2016). For reprogramming, $7.5x10^5$ skin fibroblasts were electroporated with 1 μg of each episomal vector (pCXLE-hOCT3/4-shp53-F, pCXLE-hSK, and pCXLE-hUL, all from Addgene) using the Neon System (Thermo Fisher Scientific) with the program: 1650 V, 10 msec, 3 pulses and plated onto a tissue culture-treated plastic dish for 7 days. Then, electroporated fibroblasts were trypsinized, seeded on 0.1% gelatin-coated plate with a feeder layer of irradiated mouse embryonic fibroblasts (MEFs) and cultured in stem cell medium containing knockout DMEM, 20% KO-Serum Replacement (KOSR), 1 mM NEAAs, 1% Penicillin/Streptomycin, 1% L-Glutamine, 0.1 mM β-mercaptoethanol (all from Thermo Fisher Scientific) and 10 ng/ml bFGF (Merck-Millipore), until the formation of hiPSC colonies (about 3 weeks later). Newly formed hiPSC colonies were manually picked, replated on 0.1% gelatin-coated plate with MEFs in stem cell medium. Selected hiPSC colonies were maintained on MEFs, amplified and passaged by enzymatic dissociation using 1 mg/ml Collagenase IV (Thermo Fisher Scientific) for 5–10 passages. Then, hiPSCs were adapted to feeder-free condition culturing them in Essential 8™ Medium on plates coated with Vitronectin (VTN-N) Truncated Recombinant Human Protein (all from Thermo Fisher Scientific) and expanded twice a week using 0.5 mM Ultra Pure EDTA (Thermo Fisher Scientific) with a split ratio of 1:6. Cells were kept at 37 °C, 5% CO_2, 20% O_2 in a humidified incubator.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Transmission light microscopy	Brightfield images showing the typical pluripotent human stem cell morphology	Fig. 1 panel C
Pluripotency status evidence for the described cell line	Qualitative analysis of immunofluorescence staining	Positive immunostaining of pluripotency markers: SSEA4, OCT3/4, NANOG	Fig. 1 panel D
	Quantitative analysis by flow cytometry	EURACi006-A positivity to: SSEA4 = 97.6% NANOG = 95.9% OCT3/4 = 94.1% EURACi006-A-1 positivity to: SSEA4 = 98.7% NANOG = 97.6% OCT3/4 = 97.1%	Fig. 1 panel E
Karyotype	Karyotype (G-banding) and higher-resolution, array-based assays	Normal karyotype: 46, XY for both hiPSC lines Resolution: 300–400 bands	Supplementary Fig. 1B
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site or targeted allele-specific PCR	Sanger sequencing of genomic DNA PCR product spanning the target site of PKP2 gene in exon 7	Fig. 1 panel B
	Transgene-specific PCR	N/A	N/A
Verification of the absence of random plasmid integration events	PCR/Southern	qPCR for EBNA-1 excluding the presence of episomal plasmids used during reprogramming	Supplementary Fig. 1A
Parental and modified cell line genetic identity evidence	STR analysis, microsatellite PCR (mPCR) or specific (mutant) allele seq	STR analysis	Available with the authors
		24 markers tested (Amel, D1S1656, TPOX, D2S441, D2S1338, D3S1358, FGA, D5S818, CSF1PO, D7S820, D8S1179, D10S1248, TH01, vWA, D12S391, D13S317, Penta E, D16S539, D18S51, D19S433, Penta D, D21S11, D22S1045 and DYS391) with 100% match	Available with the authors
Mutagenesis/genetic modification outcome analysis	Sequencing (genomic DNA PCR or RT-PCR product)	Sanger sequencing of genomic DNA PCR product spanning the target site of PKP2 gene in exon 7	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 analysis-	PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing	PCR on genomic DNA and Sanger sequencing showing no off-target sites	Supplementary Fig. 1C
Specific pathogen-free status	Mycoplasma	Mycoplasma testing by luminescence: Negative	Supplementary Fig. 1D
Multilineage differentiation potential	Trilineage *in vitro* differentiation by immunofluorescence analysis	Positive immunostaining of the three germ layer markers: ectodermal (NES, PAX6), mesodermal (VIM, CDX2, T) and endodermal (FOXA2, GATA4, EOMES)	Fig. 1 panel F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

3.3. CRISPR/Cas9 gene correction strategy

The heterozygous c.1643delG mutation located in exon 7 of the *PKP2* gene was corrected by insertion of a single base (G) using CRISPR/Cas9-HDR as illustrated in Fig. 1A. A guide RNA (gRNA, target site: GACATATTCTACAACGTCAC TGG) was designed using the web tool crispor.tefor.net.

CRISPR/Cas9 reagents were delivered as ribonucleoprotein (RNP) complex, composed of CRISPR/Cas9 guide RNA (crRNA:tracrRNA duplex) and the high-fidelity Cas9 nuclease protein (IDT). Synthetic RNA oligonucleotides (target site: 5′- GACATATTCTACAACGTCAC −3′, 10 pmol crRNA/tracrRNA, all from IDT) were annealed for 5 min at 95 °C in duplex buffer (IDT) and cooled down at RT for 1 h, followed by the addition of 1 μg of Cas9 protein. Moreover, 40 pmol of 140 nt ssODN (5′-TCCCCTTTTCTGGGTGGCCTGAAGGA-GACTACCCAAAAGCAAATGGTTTGCTCGATTTTGACATATTCTA-CAACGTGAC

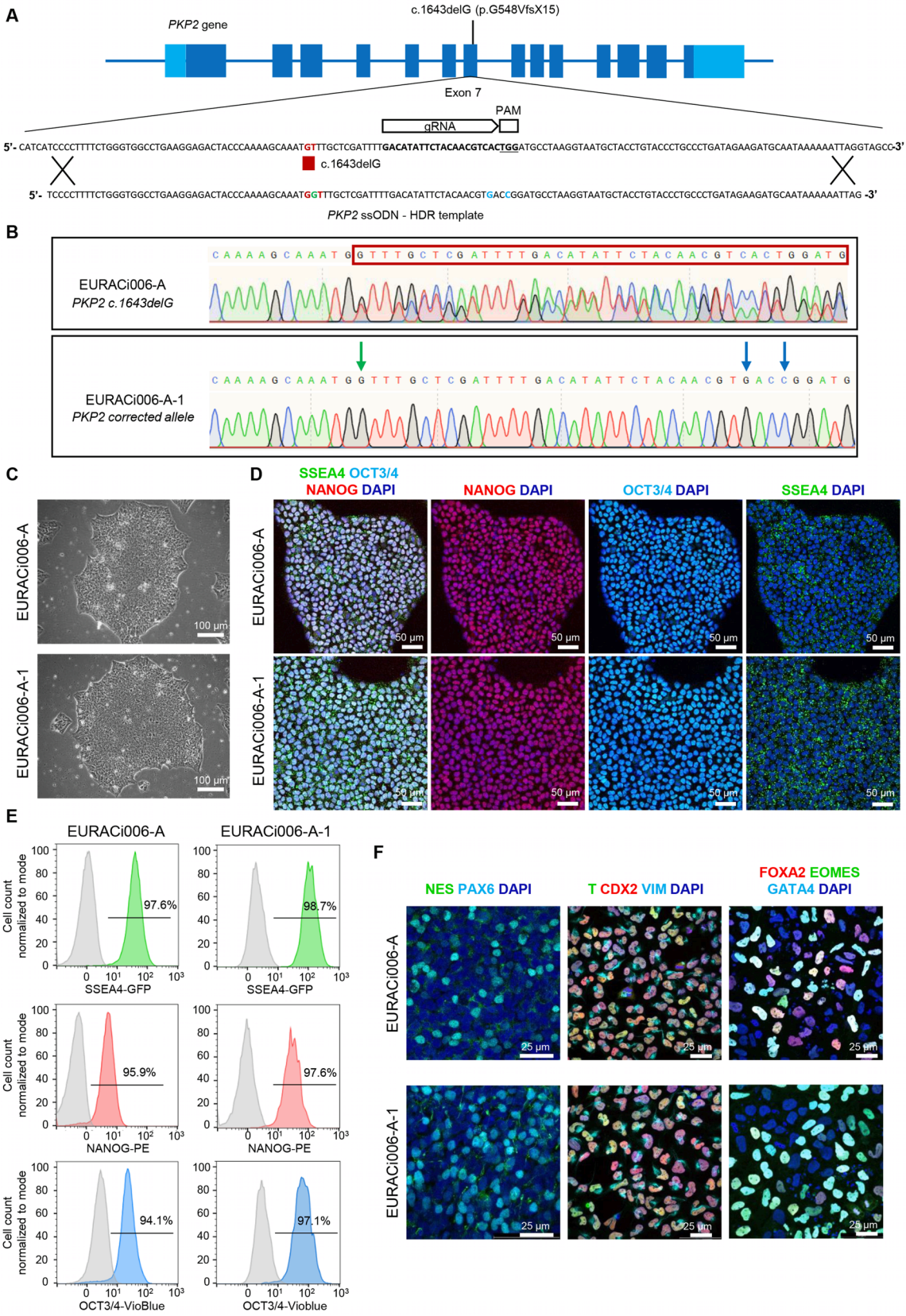
CGGATGCCTAAGGTAATGCTACCTGTACCCTGCCCTGATAGAA-GATGCAATAAAAAATTAG-3′, IDT Ultramer) was added as HDR template to correct the mutation. 3.6x10^5 hiPSCs (passage 25) were transfected with RNP complex and ssODN by electroporation using the Neon System (Thermo Fisher Scientific) using the following program: 1100 V, 20 ms, 2 pulses. After electroporation, hiPSCs were plated in Essential 8™ Medium + CloneR supplement (Stem Cell Technologies) into 12-well plate coated with Synthemax® II-SC substrate (Corning) at 37 °C, 5% CO_2, 20% O_2 in a humidified incubator. After recovering (5–7 days post electroporation), cells were plated at single cell dilution in Essential 8™ + CloneR in a 10-cm plate (100 cells/plate, 18 cells/cm^2) coated with Synthemax® II-SC substrate. Colonies emerging from single cell deposition were picked and screened for the desired base correction by Sanger sequencing of amplicon spanning the target site of *PKP2* gene in exon 7.

3.4. Immunofluorescence staining

For immunostaining, EURACi006-A (at passage 20) and EURACi006-A-1 (at passage 29) were fixed in PFA 2% for 20 min. Permeabilization and blocking of unspecific binding sites was achieved by incubation with 4% normal swine serum (NSS, DAKO) and 0.1% of Triton X-100 at RT for 1 h. Primary antibodies were incubated overnight at 4 °C, followed by incubation with secondary antibodies for 1 h in the dark at RT. Nuclei were counterstained with DAPI for 5 min in the dark at RT (Table 2). Images were acquired using a Leica TCS SP8 X microscope.

3.5. Flow cytometry analysis

EURACi006-A (at passage 20) and EURACi006-A-1 (at passage 29) were dissociated into single cells using Gentle Cell dissociation reagent (8 min at 37 °C, Stemcell Technologies) and fixed/permeabilized with the FIX & PERM™ Cell Permeabilization kit (Thermo Fisher Scientific),



(caption on next page)

Fig. 1. Generation and characterization of hiPSC line EURACi006-A from a patient affected by ACM and its isogenic corrected pair line EURACi006-A-1. (A) Schematic showing the gene correction strategy. At the top is the *PKP2* gene (blue boxes represent exons, in dark blue the coding regions, in light blue 3'- and 5'-untranslated regions); the c.1643delG (p.G548VfsX15) mutation is in exon 7. Below is the region of exon 7, showing the two bases surrounding *PKP2* c.1643delG mutation in red and the gRNA/PAM sequences (bold and underlined, respectively). At the bottom the sequence of ssODN used as HDR template is shown, with the genetic correction (G nucleotide, green) and the silent mutations (blue). (B) Sanger sequencing results showing the heterozygous *PKP2* c.1643delG in EURACi006-A (upper panel) and the correction of the *PKP2* mutation in EURACi006-A-i (lower panel). The insertion of G for PKP2 correction and the two silent mutations are indicated by green and blue arrows, respectively. (C) Representative brightfield images showing the typical pluripotent human stem cell colony morphology; scale bar: 100 μm. (D) Representative images showing positive immunofluorescence staining of pluripotency markers SSEA4 (green), OCT3/4 (cyan) and NANOG (red). Nuclei are stained with DAPI (blue); scale bar: 50 μm. (E) Flow cytometry analysis quantifying the expression of pluripotency markers SSEA4, NANOG and OCT3/4. (F) Representative immunofluorescence staining showing the expression of three germ layer markers: NES (green) and PAX6 (cyan) for ectoderm; T (green), CDX2 (red) and VIM (cyan) for mesoderm; FOXA2 (red), EOMES (green) and GATA4 (cyan) for endoderm. Nuclei are stained with DAPI (blue); scale bar: 25 μm.

according to the manufacturer's instructions. Cells were incubated with the conjugated antibodies (Table 2) for 1 h in the dark at RT and measured with a MACSQuant VYB (Miltenyi Biotech). Flow ciytometry data were analysed using FlowJo software.

3.6. In vitro trilineage differentiation

The ability of EURACi006-A (at passage 22) and EURACi006-A-1 (at passage 31) to differentiate into the three germ layers (ectoderm, mesoderm and endoderm) was assessed using the STEMdiff™ Trilineage Differentiation Kit, following the manufacturer's instructions (STEMCELL Technologies).

3.7. Karyotype analysis

Karyotype of EURACi006-A and EURACi006-A-1 was analysed at 29 and 38 passages *in vitro,* respectively. Karyotype analysis was performed using G-banding technique at 300–400 band resolution on 20 metaphase spreads (Department of Clinical Genetics, LUMC).

3.8. Sequencing

Genomic DNA used for *PKP2* sequencing was isolated from hiPSCs using the High Pure PCR Template Preparation Kit (Roche), following the manufacturer's instructions. *PKP2* mutation analysis was performed

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers (Flow cytometry)	Anti-SSEA4-FITC conjugate	1:25	Miltenyi Biotec Cat# 130-098-371 RRID:AB_2653517
Pluripotency markers (Flow cytometry)	Anti-NANOG-PE conjugate	1:5	BD Biosciences Cat# 560483, RRID:AB_1645522
Pluripotency markers (Flow cytometry)	Anti- OCT3/4-BV421 conjugate	1:25	BD Biosciences Cat# 565644, RRID:AB_2739320
Pluripotency markers (Immunocytochemistry)	Mouse IgG2b anti-OCT3/4	1:100	Santa Cruz Biotechnology Cat# sc-5279 RRID:AB_628051
Pluripotency markers (Immunocytochemistry)	Mouse IgG1 anti-NANOG	1:150	Santa Cruz Biotechnology Cat# sc-293121 RRID:AB_2665475
Pluripotency markers (Immunocytochemistry)	Mouse IgG3 anti-SSEA4	1:30	BioLegend Cat# 330402, RRID:AB_1089208
Differentiation markers (Immunocytochemistry)	Anti-PAX6-Alexa Fluor 647 Conjugate	1:200	Cell Signaling Technology Cat# 60433, RRID:AB_2797599
Differentiation markers (Immunocytochemistry)	Anti-Nestin (NES)-Alexa Fluor 488 Conjugate	1:200	Cell Signaling Technology Cat# 33475, RRID:AB_2799037
Differentiation markers (Immunocytochemistry)	Anti-Vimentin (VIM) Alexa Fluor 647 Conjugate	1:400	Cell Signaling Technology Cat# 5741, RRID:AB_10695459
Differentiation markers (Immunocytochemistry)	Anti-CDX2 Alexa Fluor 555 Conjugate	1:500	Cell Signaling Technology Cat# 12306, RRID:AB_2797879
Differentiation markers (Immunocytochemistry)	Anti-Brachyury T (T) Alexa Fluor 488 Conjugate	1:200	Cell Signaling Technology Cat# 81694, RRID:AB_2799983
Differentiation markers (Immunocytochemistry)	Anti-FOXA2 Alexa Fluor 555 Conjugate	1:500	Cell Signaling Technology Cat# 8186, RRID:AB_10891055
Differentiation markers (Immunocytochemistry)	Anti-EOMES Alexa Fluor 488 Conjugate	1:100	Cell Signaling Technology Cat# 81493, RRID:AB_2799974
Differentiation markers (Immunocytochemistry)	Anti-GATA4 Alexa Fluor 647 Conjugate	1:200	Cell Signaling Technology Cat# 36966, RRID:AB_2799108
Secondary antibodies (Immunocytochemistry)	Goat anti-Mouse IgG3 Cross-Adsorbed Secondary Antibody, Alexa 488	1:250	Thermo Fisher Scientific Cat# A21151, RRID:AB_2535784
Secondary antibodies (Immunocytochemistry)	Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody, Alexa 568	1:250	Thermo Fisher Scientific Cat# A-21124, RRID:AB_2535766
Secondary antibodies (Immunocytochemistry)	Goat anti-Mouse IgG2a Cross-Adsorbed Secondary Antibody, Alexa 647	1:250	Thermo Fisher Scientific Cat# A-21242, RRID:AB_2535811
Nuclear stain	DAPI	5 μg/ml	Thermo Fisher Scientific Cat# D3571, RRID:AB_2307445
Site-specific nuclease			
Nuclease information	Recombinant Cas9 protein	Integrated DNA Technologies, IDT Cat# 1081066	
Delivery method	RNP electroporation	Electroporation program: 1100 V, 20 ms, 2 pulses	
Selection/enrichment strategy	N/A	N/A	

Primers and Oligonucleotides used in this study		
	Target	Forward/Reverse primer (5′-3′)
Targeted mutation analysis (PCR/sequencing)	*PKP2* exon 7	GCATGTCTGGCCTTTATTTG/CCAGCCAAGGGTTTTCTTAT
Episomal plasmid exclusion (qPCR)	*EBNA1*	ATCAGGGCCAAGACATAGAGATG/GCCAATGCAACTTGGACGT
Housekeeping gene plasmid exclusion (qPCR)	*FBXO15*	GCCAGGAGGTCTTCGCTGTA/AATGCACGGCTAGGGTCAAA
Genomic target sequence	gRNA including PAM sequence (bold)	GACATATTCTACAACGTCAC **TGG**
ssODN used as template for HDR-mediated site-directed mutagenesis	ssODN	TCCCCTTTTCTGGGTGGCCTGAAGGAGACTACCCAAAAGCAA ATGGTTTGCTCGATTTTGACATATTCTACAACGTGACCGGATGC CTAAGGTAATGCTACCTGTACCCTGCCCTGATAGAAGATGCAAT AAAAAATTAG
Top off-target mutagenesis predicted site sequencing	*OT1 (HDAC2)*	AGCACGGAGAAGAGAGAAGC/GCAGCCACCATTCTGCTTAC
	OT2 (RHOBTB2)	AAGGGTCAGCTCCCAAAGAG/ACTAGGCACAGAGCTGGAAC
	OT3 (STAB2)	CTAAAGCAACCTCCCCTGGT/CTGGCTTTGTGCTGATGCTT
	OT4 (PKP3)	ACCCTGTCCTGTGGTTTTGT/TAGAGGCGGTAGGACAGGTT
	OT5 (SHANK2)	TTTCCGGCATTGTTTCAGGA/TGTCTGATTGCATGGGGATG

on a PCR product obtained by genomic DNA amplification using Platinum™ Taq DNA Polymerase High Fidelity (Invitrogen), following manufacturer's instructions (primers are listed in Table 2). The PCR reaction was run using the following conditions: 94 °C 1 min/94 °C 30 s; 58 °C 30 s; 68 °C 1 min for 35 cycles/68 °C 5 min; 12 °C ∞. The PCR product of 885 bp was purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced (primers in Table 2).

3.9. Off-target analysis

In silico predicted off-target sites cleavage by CRISPR/Cas9 were identified using the online tool crispor.tefor.net. Off-target analysis on the top-5 *in silico* predicted off-target sites located in coding regions was performed by Sanger sequencing of the PCR products obtained using the primers listed in Table 2.

3.10. qPCR to exclude the presence of episomal plasmids

The presence of episomal plasmid integration was evaluated by episomal specific primers for EBNA-1. Primers are shown in Table 2. Skin fibroblasts 5 days after transfection, with strong transgene expression level are used as positive control. Skin fibroblasts never exposed to reprogramming plasmids are used as negative control. Genomic DNA from parental skin fibroblasts and hiPSC lines (EURACi006-A at passage 18 and EURACi006-A-1 at passage 27) was extracted using High Pure PCR Template Preparation Kit (Roche), following the manufacturer's instruction. iTaq Universal SYBR Green Supermix was used for DNA amplification on CFX96 Real-Time PCR Detection System (all from BioRad) using the following conditions: 95 °C 3 min/95 °C 30 s; 60 °C 1 min for 39 cycles/95 °C 10 s; melt curve 60 °C to 95 °C with increment 0.5 °C.

3.11. STR analysis

Cell identity was assessed on genomic DNA from both hiPSC lines and parental skin fibroblasts with the Promega PowerPlex Fusion System 5C autosomal STR kit (Westen et al. 2014).

3.12. Mycoplasma test

MycoAlert™ Mycoplasma Detection Kit (Lonza), following manufacturer's instructions, was used to test the absence of Mycoplasma in EURACi006-A and EURACi006-A-1 at 29 and 38 passages *in vitro*, respectively (Table 1).

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.

Acknowledgements

We thank P. de Knjiff (Dept. of Human Genetics, LUMC) for STR analysis. This work was supported by the Department of Innovation and Research and University of the Autonomous Province of Bolzano/Bozen, the Transnational Research Project on Cardiovascular Diseases (JTC2016_FP-40-021 ACM-HF) and the Netherlands Organisation for Health Research and Development ZonMW (MKMD project no. 114022504).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2021.102426.

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