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Lab Resource: Multiple Cell Lines

Generation and characterization of three human induced pluripotent stem cell lines (EURACi007-A, EURACi008-A, EURACi009-A) from three different individuals of the same family with arrhythmogenic cardiomyopathy (ACM) carrying the plakophillin2 p.N346Lfs*12 mutation

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A B S T R A C T

Arrhythmogenic Cardiomyopathy (ACM) is a genetically based cardiomyopathy associated with ventricular arrhythmias and fibro-fatty substitution of cardiac tissue. It is characterized by incomplete penetrance. We generated human iPSCs by episomal reprogramming of blood cells from three members of the same family: the proband, affected by ACM and carrying the heterozygous plakophillin2 p.N346Lfs*12 mutation, one asymptomatic carrier of the same mutation and one apparently healthy control. hiPSCs were characterized according to standard protocols including karyotyping, pluripotency marker expression and differentiation towards the three germ layers. These hiPSC lines can be used to study the mechanisms of ACM incomplete penetrance *in vitro*.

1. Resource Table:

Unique stem cell lines identifier	1. EURACi007-A 2. EURACi008-A 3. EURACi009-A
Alternative name(s) of stem cell lines	1. PZE#8 (EURACi007-A) 2. PZG#4 (EURACi008-A) 3. PZB#4 (EURACi009-A)
Institution	Institute for Biomedicine, Eurac Research, Bolzano, Italy
Contact information of distributor	Alessandra Rossini (alessandra.rossini@eurac.edu)
Type of cell lines	iPSCs
Origin	Human
Additional origin info required *for human ESC or hiPSC*	1. EURACi007-A Age: 31 Sex: Female Ethnicity if known: Caucasian 2. EURACi008-A Age: 67 Sex: Female Ethnicity if known: Caucasian 1. EURACi009-A Age: 65 Sex: Female Ethnicity if known: Caucasian
Cell Source	Peripheral blood mononuclear cells (PBMNCs)
Clonality	Clonal
Method of reprogramming	Non-integrating episomal vectors (pCXLE hOCT3/4-shp53-F, pCXLE-hSK, and pCXLE-hUL)
Genetic Modification	YES for lines EURACi007-A and EURACi008-A, NO for line EURACi009-A
Type of Genetic Modification	Spontaneous mutation in EURACi007-A and EURACi008-A
Evidence of the reprogramming transgene loss	qPCR to exclude the presence of episomal plasmids
Associated disease	Arrhythmogenic Cardiomyopathy (OMIM: 609040)
Gene/locus	*PKP2*/12p11.21 Heterozygous *deletion of 9993 bp encompassing exon 4*,c.1035-5203_1171-13298del; p.N346Lfs*12
Date archived/stock date	

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(*continued*)

	November 2015 (EURACi007-A) October 2017 (EURACi008-A) November 2015 (EURACi009-A)
Cell line repository/bank	https://hpscreg.eu/cell-line/EURACi007-A; https://hpscreg.eu/cell-line/EURACi008-A; https://hpscreg.eu/cell-line/EURACi009-A
Ethical approval	Blood cells were collected after patient signed informed consent and the protocol hiPSC generation was approved by the Ethical Committee of the Province of South Tyrol (Nr.1/2014, 12/03/2014).

2. Resource utility

The three hiPSC lines EURACi007-A (PKP2-mutated symptomatic), EURACi008-A (PKP2-mutated asymptomatic) and EURACi009-A (healthy control) obtained from members of the same family (partly sharing genetic information) not only offer the possibility to study ACM pathogenesis and test new drugs but represent a tool to investigate the disease mechanisms of incomplete penetrance.

3. Resource details

Arrhythmogenic cardiomyopathy (ACM, OMIM: 609040) is a rare inherited cardiac disease. Causative ACM variants are mainly located in genes encoding proteins of cardiac desmosomes, with *PKP2* being the most common casual gene (James et al. 2020). The disease phenotype is characterised by electrical abnormalities, often life-threatening, and progressive fibro-fatty substitution of the cardiac tissue. However, the manifestations of the disease are highly variable, and the penetrance of mutations associated with ACM is often incomplete (Hoorntje et al. 2017). Sex (Akdis et al. 2017) and endurance exercise (Zorzi et al. 2021) have been related to ACM incomplete penetrance; however its molecular and cellular basis are far from being understood. Therefore, the three hiPSC lines that we generated could contribute to shed light on this complex phenomenon. We generated three hiPSC lines from individuals belonging to the same family: the EURACi007-A line from a 31 year old female carrying a heterozygous deletion in *PKP2* gene (c.1035-5203_1171-13298del) that leads to the loss of whole exon 4 (breakpoints within introns 3 and 4), with a consequent frameshift and the creation of a premature stop codon (p.N346Lfs*12); the EURACi008-A line from a 67 year old female carrying the same *PKP2* mutation, but showing no symptoms of the disease, and the EURACi008 line from a 61 year old female without *PKP2* mutation (Fig. 1A). For each individual, we produced several hiPSC clones by reprogramming of PBMNCs using electroporation of episomal plasmids carrying *OCT3/4*, *SOX2*, *KLF4*, and *L-MYC* (Meraviglia et al. 2015) and one single clone per individual was randomly selected based on morphology and characterized as reported in Table 1. Each clone of all the three lines showed the typical human embryonic-stem cell-like morphology with high nuclear/cytoplasmatic ratio (Fig. 1B). Pluripotency was assessed by immunofluorescence staining for SOX2, OCT4 (nuclear proteins), SSEA4 (membrane protein) (Fig. 1C) and by qRT-PCR, showing higher expression levels of pluripotency genes *OCT4, SOX2, NANOG* in all three hiPSC lines compared to their PBMNC counterpart (Fig. 1D). The integration of episomal plasmids was excluded in all the three hiPSC lines by qPCR, indicating the successful activation of endogenous pluripotent genes (Supplementary Fig. 1A). The potential to differentiate into all three germ layers following embryoid body (EB) formation was evaluated by qRT-PCR on endodermal (*SOX7* and *AFP* in green), mesodermal (*CD31, ACTA2, SCL* and *CDH5* in red) and ectodermal (*KTR14, NCAM1, TH* and *GABRR2* in blue) genes (Fig. 1E). The presence of the heterozygous *PKP2* deletion in EURACi007-A and EURACi008-A and the absence of this mutation in EURACi009-A was confirmed by Sanger sequencing (Fig. 1F). STR analysis for 16 short tandem repeat markers (Table 1) showed identical profiles for hiPSC lines with their parental blood cell line. Q-banding cytogenetic analysis was performed on EURACi007-A (at passage 42), EURACi008-A (at passage 30) and EURACi009-A (at passage 65), showing normal karyotype and no clonal abnormalities (Supplementary Fig. 1B). hiPSC lines were also tested with a standard Mycoplasma assay (MycoAlert™) and resulted negative (Supplementary Fig. 1C).

4. Materials and methods

4.1. Ethical statemen

Blood was collected after patient written informed consent. This study was approved by the Ethical Committee of the Province of Alto Adige/South Tyrol (Nr.1/2014, 12/03/2014).

4.2. Cell culture and reprogramming

PBMNCs isolated from venous peripheral blood were amplified as previously described (Meraviglia et al. 2015). PBMNCs (5×10^6) were electroporated with 1 μg of each episomal vector (pCXLE-hOCT3/4-shp53-F, pCXLE-hSK, and pCXLE-hUL, all from Addgene) using Neon System (Thermo Fisher Scientific) following the program: 1650 V, 10 ms, 3 pulses and then PBMNCs were plated on a feeder layer of irradiated mouse embryonic fibroblasts (MEFs) three days after electroporation. Transfected PBMNCs were cultured in hiPSC medium containing knockout (KO) DMEM, 20% KO-Serum Replacement, 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 (approximately 20–30 days later). Selected hiPSC colonies were maintained on MEFs for 5–10 passages and amplified by enzymatic dissociation using 1 mg/ml Collagenase IV (Thermo Fisher Scientific). Then, hiPSCs were adapted to feeder-free condition by culturing the cells in StemMACS™ iPS-Brew XF (Miltenyi Biotec) on Matrigel matrix (0.083 mg/ml, Corning) and expanded using 1X TrypLE™ (Thermo Fisher Scientific) twice a week with a split ratio of 1:5. Y-27632 (10 μM, Sigma-Aldrich) was used after passaging to improve cell recovery. Cells were kept at 37 °C, 5% CO_2, 20% O_2 in a humidified incubator.

4.3. Immunofluorescence staining

The detection of pluripotency markers on undifferentiated hiPSCs (all the three lines at passage 7) was assessed using Pluripotent Stem Cell Marker Immunocytochemistry Kit (Thermo Fisher Scientific), according to manufacturer's instructions (Table 2). Nuclei were counterstained with DAPI.

4.4. In vitro trilineage differentiation

To assess their ability to form three germ layers, hiPSCs (all the three lines at passage 6) were cultured in suspension in ultra-low attachment plates for 7 days to form EBs using EB 20% medium composed of KO-DMEM (Thermo Fisher Scientific) with 20% FBS Defined (Hyclone), 1 mM NEAAs, 1% Penicillin/Streptomycin, 1% L-Glutamine, 0.1 mM β-mercaptoethanol (all from Thermo Fisher Scientific). EBs were then plated onto 0.1% gelatin-coated dishes and cultured in EB 20% medium for further 15–20 days of differentiation. EBs were kept at 37 °C, 5% CO_2, 20% O_2 in a humidified incubator.

4.5. qRT-PCR

Total RNA from PBMNCs, hiPSCs (all the three lines at passage 6) and EBs was extracted using TRIzol® Reagent and cDNA was obtained by reverse transcription of RNA (1 μg) using SuperScript VILO cDNA Synthesis Kit following manufacturer's instruction (all from Thermo Fisher Scientific). All-in-One SYBR® Green qPCR Mix (GeneCopoeia) was used

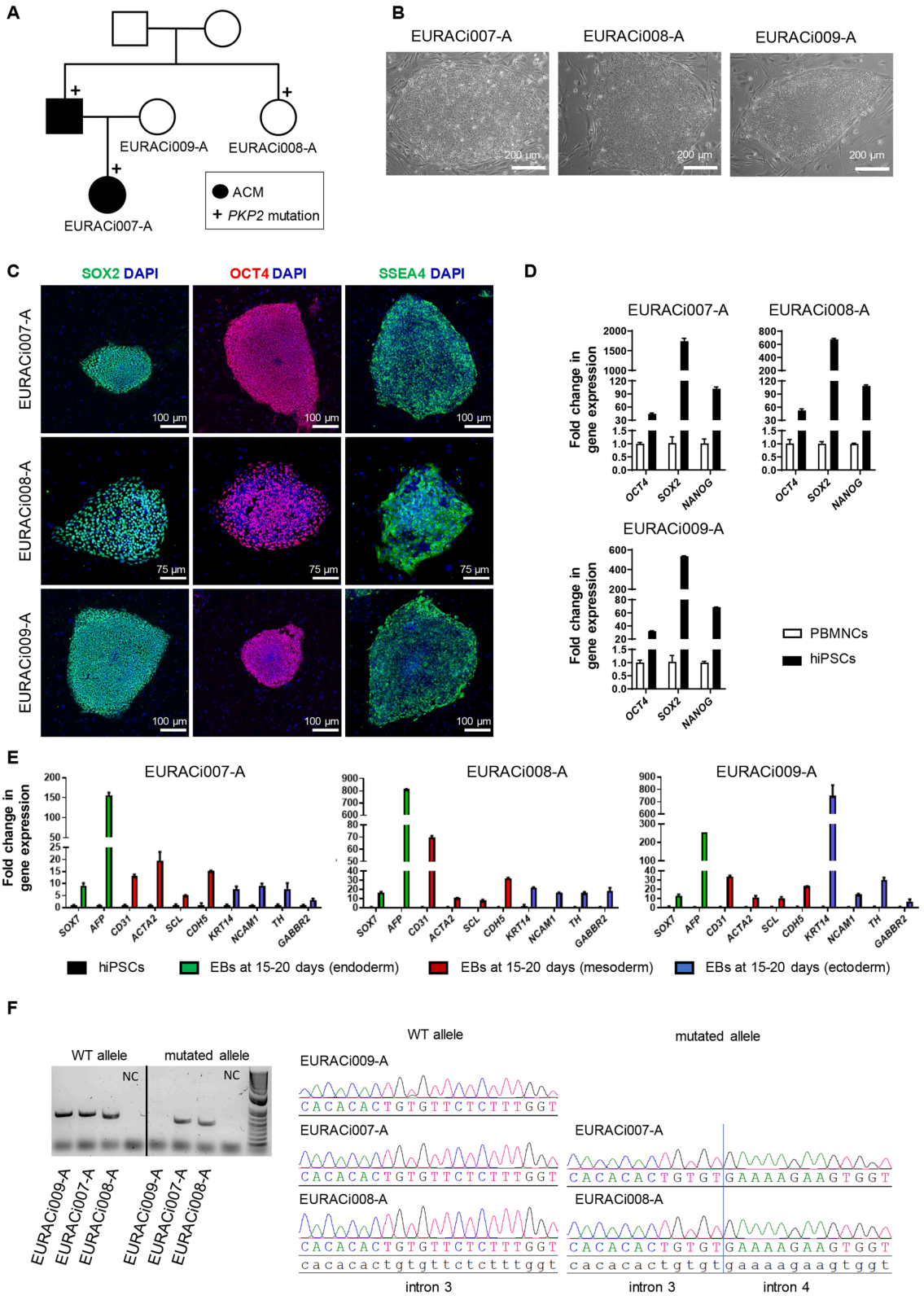

Fig. 1. Generation and characterization of hiPSC lines EURACi007-A, EURACi008-A and EURACi009-A belonging to the same family. (A) Pedigree of the ACM family carrying a heterozygous PKP2 mutation. EURACi007-A is the proband, EURACi008-A is an asymptomatic mutation carrier and EURACi009-A is a healthy family member. (B) Representative brighfiled images showing the hiPSC colony morphology; scale bar: 100 µm. (C) Representative immunofluorescence staining showing positive staining of pluripotency proteins SOX2 (green), OCT4 (red) and SSEA4 (green). Nuclei are counterstained with DAPI; scale bar 75 µm-100 µm. (D) Gene expression analysis indicating the re-expression of endogenous OCT4, SOX2, NANOG pluripotency genes. (E) qRT-PCR analysis of three germ layer genes after 15-20 days of hiPSC differentiation via embryoid body formation. (F) Sanger sequencing results showing the presence of the wildtype (WT) allele in all 3 hiPSC lines, and the presence of the mutated allele only in EURACi007-A, EURACi008-A lines.

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 B
Phenotype	Qualitative analysis *(Immunocytochemistry)*	Positive immunostaining of pluripotency markers: SSEA4, OCT4, SOX2	Fig. 1 panel C
	Quantitative analysis *(RT-qPCR)*	Fold change for pluripotency genes: EURACi007-A OCT4 = 44.5 ± 2.4 SOX2 = 1741.7 ± 102.4 NANOG = 101.9 ± 5.5 EURACi008-A OCT4 = 52.6 ± 4.9 SOX2 = 675.7 ± 19.9 NANOG = 108.8 ± 3.2 EURACi009-A OCT4 = 32.1 ± 0,94 SOX2 = 535.6 ± 5.2 NANOG = 68.6 ± 0.33	Fig. 1 panel D
Genotype	Karyotype (Q-banding)	Normal karyotype: 46, XX for all the three iPSC lines Resolution: 300–400 bands	Supplementary Fig. 1B
Identity	Microsatellite PCR (mPCR) OR STR analysis	N/A The following 16 markers were tested: CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, TH01, TPOX, vWA and the gender marker Amelogenin. 100% match for all the three hiPSC lines	N/A Submitted in archive with journal
Mutation analysis	Sequencing	Heterozygous *PKP2* c.1035-5203_1171-13298del (EURACi007-A) Heterozygous *PKP2* c.1035-5203_1171-13298del (EURACi008-A) Wildtype *PKP2* (EURACi009-A)	Fig. 1 panel F
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by luminescence: Negative	Supplementary Fig. 1C
Differentiation potential	Embryoid body formation	Expression of specific genes for the three germ layers in embryoid bodies	Fig. 1 panel E
List of recommended germ layer markers	Expression of these markers has to be demonstrated at mRNA (qRT-PCR)	Ectoderm*: KRT14, NCAM1, TH* and *GABBR2* Endoderm: *SOX7* and *AFP* Mesoderm: *CD31, ACTA2, SCL* and *CDH5*	qRT-PCR with reference gene

for cDNA amplification on CFX96 Real-Time PCR Detection System (BioRad) using the following conditions: 95 °C 10 min/95 °C 15 s; 60 °C 45 s min for 44 cycles/95 °C 1 min; melt curve 55 °C to 95 °C with increment 0.5 °C every 5 s. Primers are reported in Table 2.

4.6. Karyotype analysis

Karyotype of EURACi007-A, EURACi008-A and EURACi009-A-1 was analysed at 42, 30 and 65 passages *in vitro,* respectively. Karyotype analysis was performed using Q-banding technique at 300–400 band resolution on 20 metaphase spreads, as previously described (Meraviglia et al. 2015).

4.7. Sanger sequencing

Genomic DNA used for *PKP2* sequencing was isolated from hiPSCs using the QIAamp DNA Blood Kits (Qiagen), following the manufacturer's instructions. We have designed specific primers to amplify only the wildtype allele at the breakpoint within intron 3 and specific primers to amplify only the mutated allele (Table 2). The heterozygous *PKP2* mutation analysis was performed on the two PCR products obtained by genomic DNA amplification using Q5 High-Fidelity 2X Master Mix (Euroclone), following manufacturer's instructions. The PCR reactions were run on Mastercycler®pro S instrument (Eppendorf) using the following conditions: 98 °C 30 s/98 °C 10 s; 63 °C 20 s; 72 °C 30 s for 30 cycles/72 °C 1 min; 4 °C 10 min. The expected product sizes for wild type allele and mutated allele are 668 bp and 485 bp, respectively. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced with the same primer Forward (Table 2). The sequencing was performed by Eurofins Genomics (Germany).

4.8. qPCR to exclude the presence of episomal plasmids

Genomic DNA from parental PBMNCs and hiPSC lines (all the three lines at passage 6) was extracted using DNeasy Blood and Tissue kit (Qiagen), following manufacturer's instructions. All-in-One SYBR® Green qPCR Mix (GeneCopoeia) 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 every 5 s. PBMNCs 5 days after electroporation, with strong transgene expression level are used as positive control, while PBMNCs never exposed to reprogramming plasmids are used as negative control. The presence of episomal vectors used for reprogramming was evaluated by transgene specific primers for EBNA-1 (Table 2).

4.9. STR analysis

Cell identity was assessed on genomic DNA from all the three hiPSC lines and parental PBMNCs with the Applied Biosystems™ AmpFLSTR™ Identifier™ Plus PCR Amplification Kit, under ISO 17,025 accredited conditions (Eurofins Genomics). The kit includes the following STR markers: CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433,

Table 2
Reagents details.

Antibodies used for immunocytochemistry				
	Antibody	**Dilution**	**Company Cat #**	**RRID**
Pluripotency Markers (Immunocytochemistry)	Rabbit anti-OCT4	1:100	Thermo Fisher Scientific Cat# A24867	RRID:AB_2650999
Pluripotency Markers (Immunocytochemistry)	Mouse anti-SSEA4 (IgG3)	1:100	Thermo Fisher Scientific Cat# A24866	RRID:AB_2651001
Pluripotency Markers (Immunocytochemistry)	Rat anti-SOX2	1:100	Thermo Fisher Scientific Cat# A24759	RRID:AB_2651000
Secondary antibodies (Immunocytochemistry)	Alexa Fluor® 555 Donkey Anti-Rabbit	1:250	Thermo Fisher Scientific Cat# A24869	RRID:AB_2651006
Secondary antibodies (Immunocytochemistry)	Alexa Fluor® 488 Goat Anti-Mouse IgG3	1:250	Thermo Fisher Scientific Cat# A24877	RRID:AB_2651008
Secondary antibodies (Immunocytochemistry)	Alexa Fluor® 488 Donkey Anti-Rat	1:250	Thermo Fisher Scientific Cat# A24876	RRID:AB_2651007
Nuclear stain	DAPI	5 μg/ml	Thermo Fisher Scientific Cat# D3571	RRID:AB_2307445

	Primers		
	Target	**Size of band**	**Forward/Reverse primer (5′-3′)**
Pluripotency Markers (qRT-PCR)	*SOX2*	151 bp	GGGAAATGGGAGGGGTGCAAAAGAGG/ TTGCGTGAGTGTGGATGGGATTGGTG
Pluripotency Markers (qRT-PCR)	*OCT4*	144 bp	GACAGGGGGAGGGGAGGAGCTAGG/ CTTCCCTCCAACCAGTTGCCCCAAAC
Pluripotency Markers (qRT-PCR)	*NANOG*	116 bp	TGCAAGAACTCTCCAACATCCT/ ATTGCTATTCTTCGGCCAGTT
Three germ layer markers (endoderm) (qRT-PCR)	*SOX7*	112 bp	TGAACGCCTTCATGGTTTG/ AGCGCCTTCCACGACTTT
Three germ layer markers (endoderm) (qRT-PCR)	*AFP*	101 bp	GTGCCAAGCTCAGGGTGTAG/ CAGCCTCAAGTTGTTCCTCTG
Three germ layer markers (mesoderm) (qRT-PCR)	*CD31*	108 bp	ATGCCGTGGAAAGCAGATAC/ CTGTTCTTCTCGGAACATGGA
Three germ layer markers (mesoderm) (qRT-PCR)	*ACTA2*	112 bp	GTGATCACCATCGGAAATGAA/ TCATGATGCTGTTGTAGGTGGT
Three germ layer markers (mesoderm) (qRT-PCR)	*SCL*	98 bp	CCAACAATCGAGTGAAGAGGA/ CCGGCTGTTGGTGAAGATAC
Three germ layer markers (mesoderm) (qRT-PCR)	*CDH5*	73 bp	GAGCATCCAGGCAGTGGTAG/ CAGGAAGATGAGCAGGGTGA
Three germ layer markers (ectoderm) (qRT-PCR)	*KRT14*	86 bp	CACCTCTCCTCCTCCCAGTT/ ATGACCTTGGTGCGGATTT
Three germ layer markers (neuro-ectoderm) (qRT-PCR)	*NCAM1*	136 bp	CAGATGGGAGAGGATGGAAA/ CAGACGGGAGCCTGATCTCT
Three germ layer markers (neuro-ectoderm) (qRT-PCR)	*TH*	120 bp	TGTACTGGTTCACGGTGGAGT/ TCTCAGGCTCCTCAGACAGG
Three germ layer markers (neuro-ectoderm) (qRT-PCR)	*GABRR2*	106 bp	CTGTGCCTGCCAGAGTTTCA/ ACGGCCTTGACGTAGGAGA
House-Keeping Gene (qRT-PCR)	*GAPDH*	89 bp	CCACCCATGGCAAATTCC/ TCGCTCCTGGAAGATGGTG
Episomal plasmid exclusion (qPCR)	*EBNA1*	61 bp	ATCAGGGCCAAGACATAGAGATG/ GCCAATGCAACTTGGACGT
Housekeeping gene plasmid exclusion (qPCR)	*FBXO15*	100 bp	GCCAGGAGGTCTTCGCTGTA/ AATGCACGGCTAGGGTCAAA
Targeted mutation analysis (PCR/sequencing)	*PKP2 wt allele*	668 bp	PCR: GGAACACTTGGGGTCAAGAA/CAGCCTCCAACTGGTCATAAA Sequencing: GGAACACTTGGGGTCAAGAA
Targeted mutation analysis (PCR/sequencing)	*PKP2 mutated allele*	485 bp	PCR: GGAACACTTGGGGTCAAGAA/CCAACATCCATTCGTGATTC Sequencing: GGAACACTTGGGGTCAAGAA

D21S11, FGA, TH01, TPOX, vWA, and the gender marker Amelogenin.

4.10. Mycoplasma test

The MycoAlert™ Mycoplasma Detection Kit (Lonza), following manufacturer's instructions, was used to test the absence of Mycoplasma in EURACi007-A, EURACi008-A and EURACi009-A at 42, 30 and 51 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

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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.2021.102466.

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