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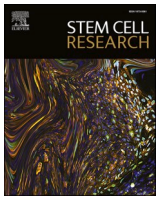

Lab Resource: Single Cell Line

Generation of human induced pluripotent stem cell line EURACi015-A from a patient affected by dilated cardiomyopathy carrying the Lamin A/C p. Glu161Lys mutation

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A B S T R A C T

Dilated cardiomyopathy (DCM) is a common heart disorder caused by genetic and non-genetic etiologies, characterized by left ventricular dilatation and contractile dysfunction. Here, we created a human induced pluripotent stem cell line from peripheral blood mononuclear cells using non-integrating vectors from a patient carrying a heterozygous *LMNA* variant (c.481G > A, p.Glu161Lys, NM_170707.4). The obtained EURACi015-A line, showed the typical morphology of pluripotent cells, normal karyotype and exhibited pluripotency markers and a trilineage differentiation potential. This cell line can be successfully differentiated into cardiomyocytes and endothelial cells. This line represents a human *in vitro* model to study the genetic basis of DCM.

1. Resource table

Unique stem cell line identifier	EURACi015-A
Alternative name(s) of stem cell line	LMNA #9
Institution	Institute for Biomedicine, Eurac Research, Bolzano, Italy
Contact information of distributor	Giada Cattelan (giada.cattelan@eurac.edu)
Type of cell line	hiPSC
Origin	Human
Additional origin info required for human ESC or iPSC	Age: 56 Sex: male Ethnicity if known: Caucasian
Cell Source	Peripheral blood mononuclear cells (PBMNCs)
Clonality	Clonal

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(*continued*)

Unique stem cell line identifier	EURACi015-A
Method of reprogramming	Non-integrating episomal vectors (pCXLE hOCT3/4-shp53-F, pCXLE-hSK and pCXLE-hUL)
Genetic Modification	YES
Type of Genetic Modification	Spontaneous mutation
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	qRT-PCR to exclude the presence of episomal plasmids
Associated disease	Dilated Cardiomyopathy (OMIM: 115200)
Gene/locus	LMNA 1q22 Heterozygous missense mutation in exon 2: c.481G > A p.Glu161Lys (NM_170707.4)
Date archived/stock date	April 2023

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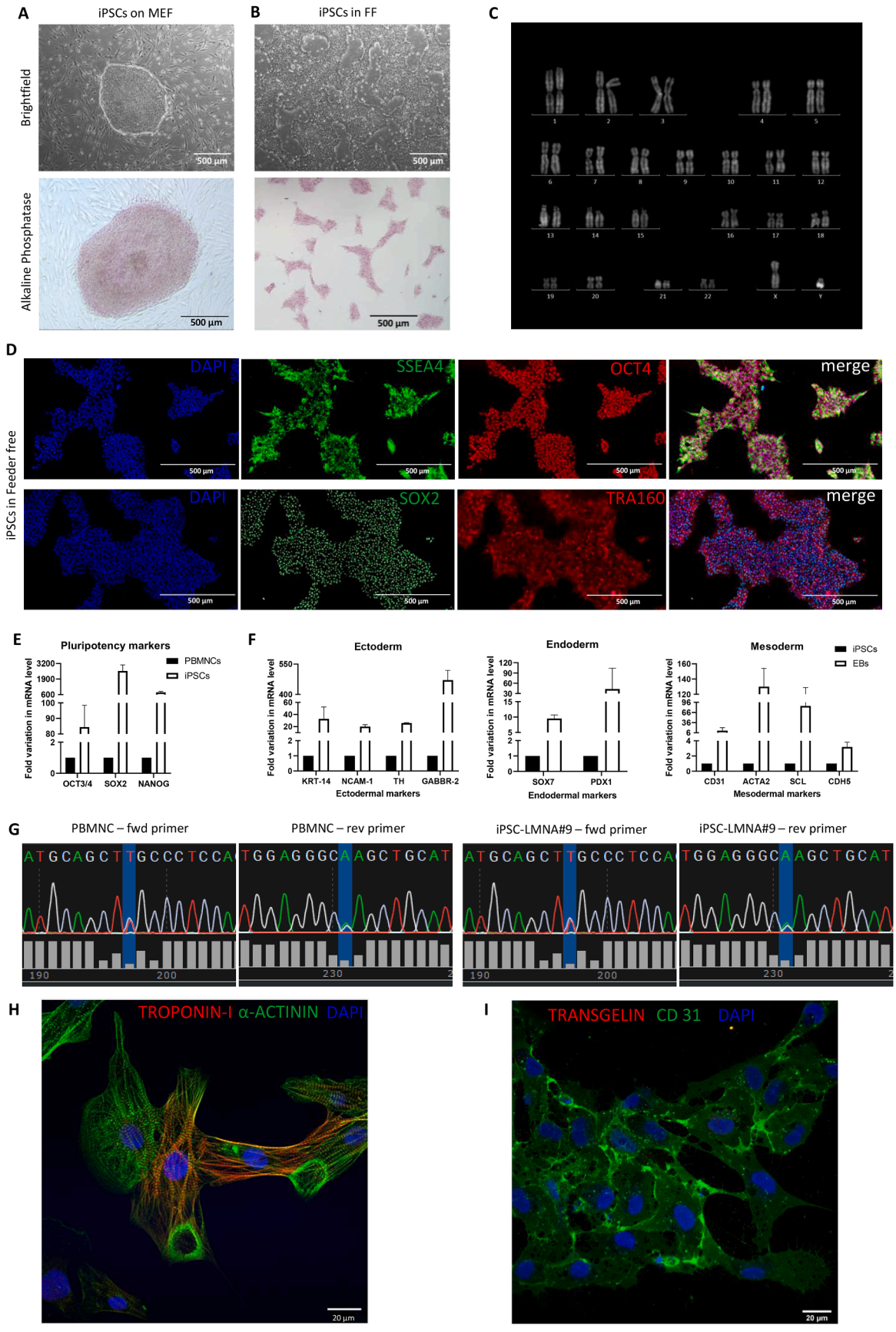

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Fig. 1. Generation and characterization of EURACi015-A hiPSC line. (A) Representative images of the morphology of hiPSC colony on a MEF layer in brightfield and positive to alkaline phosphatase; scale bar 500 μm. (B) Representative images of the morphology of hiPSC in feeder free in brightfield and positive to alkaline phosphatase; scale bar 500 μm. (C) Representative picture of normal karyotype by Q-banding karyotype analysis. (D) Representative immunofluorescence staining showing positive staining of pluripotency proteins SSEA4 (green), OCT4 (red), SOX2 (green) and TRA-1-60 (red). Nuclei are counterstained with DAPI; scale bar 500 μm. (E) Gene expression analysis indicating the expression of endogenous OCT3/4, SOX2 and NANOG pluripotency genes. (F) Gene expression analysis of three germ layer genes after 25 days of hiPSC differentiation via embryoid body formation. (G) Sanger sequencing results confirming the heterozygous missense mutation *LMNA* mutation. (H) Representative immunofluorescence images for cardiac sarcomeric proteins α-Actinin (green) and Troponin I (red). Nuclei are counterstained with DAPI; scale bar 20 μm. (I) Representative immunofluorescence images of hiPSC-derived endothelial cells resulted positive for CD31 (green) and negative for the muscle cell marker Transgelin (red). Nuclei are counterstained with Hoechst; scale bar 200 μm.

(*continued*)

Unique stem cell line identifier	EURACi015-A
Cell line repository/bank	https://hpscreg.eu/cell-line/EURACi015-A
Ethical approval	Blood cells were collected after patient signed informed consent and the protocol hiPSC generation was approved by the Regional FVG Ethical Committee (CERU N.O. 43/2009, Em02/2022)

2. Resource utility

The EURACi015-A hiPSC line, derived from a patient with the heterozygous Lamin A/C missense mutation p.Glu161Lys, represents an unlimited *in vitro* source to investigate the molecular mechanisms of the cardiac dysfunction in DCM and the possibility to develop new *in vitro* treatments treat this pathology.

3. Resource details

Dilated cardiomyopathy (DCM) is a common heart disorder with an estimated prevalence from 1:250 to 1:2500 in the general population (Orphanou et al., 2022). It is characterized by left ventricular dilatation and systolic dysfunction. About 35% of the cases are genetically inherited due to mutations affecting genes encoding sarcomere, cytoskeleton and nuclear envelope proteins. The remaining cases can be attributed to acquired conditions, including myocarditis, metabolic disorders, alcohol and drug abuse (Weintraub et al., 2017). The generation of this cell line will be precious for the elucidation of the genetic mechanisms that lead to DCM in patients carrying *LMNA* variants. These cells were generated from peripheral blood mononuclear cells (PBMNCs) harvested from a patient affected by severe familial form of DCM, with heart transplant at the age of 50 years old after a long medical history characterized by multiple hospitalization for heart failure and life threatening ventricular arrhythmias, and carrying an heterozygous pathogenic missense variant of *LMNA* gene. *LMNA* variants are cause of a particular form of arrhythmic DCM, characterized by variable expressivity of early-onset atrioventricular blocks, supraventricular and major ventricular arrhythmia (Paldino et al., 2022). PBMNCs were reprogrammed with non-integrating episomal vectors, encoding for OCT3/4, SOX2, KLF4, and L-MYC transcription factors. The generated hiPSCs were first cultured on a mouse embryonic fibroblast (MEF) feeder layer (Fig. 1A), then selected and adapted to feeder-free conditions, as shown in Fig. 1B.

Fig. 1A shows the morphology of the obtained hiPSCs colonies with their typical well-defined margins and a high nucleus/cytoplasm ratio. This cell line was strongly positive for alkaline phosphatase (Fig. 1A–B), it was negative in the mycoplasma test (Supplementary Table 1) and displayed a normal XY karyotype (Fig. 1C).

The pluripotency was evaluated by immunostaining for the pluripotent markers OCT3/4 (nuclear), SOX2 (nuclear), TRA-1-60 (surface) and SSEA4 (surface) (Fig. 1D). Further confirmation of the pluripotency state was obtained by qRT-PCR, which showed high expression levels of the pluripotency genes *OCT4, SOX2* and *NANOG* in hiPSCs compared with parental PBMNCs (Fig. 1E). The potential of this cell line to

Table 1
Characterization and validation.

Classification	Test	Result	Data
Phenotype	Qualitative analysis	Expression of pluripotency protein markers: SSEA4, OCT4, TRA-1–60, SOX2	Fig. 1D
	Quantitative analysis	Fold change for pluripotency genes: OCT4 = 84.45 ± 14 SOX2 = 2581.19 ± 502.82 NANOG = 794.26 ± 77.73	Fig. 1E
Genotype	Karyotype (Q-banding) and resolution	Normal karyotype: 46,XY Resolution: 300–400 bands	Fig. 1C
Identity	Microsatellite PCR (mPCR) OR STR analysis	N/A	N/A
		100% identical. The following markers were tested: D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, AMEL, D5S818, FGA, D19S433, vWA, TPOX and D18S51	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	Heterozygous *LMNA* mutation: c.481G > A (NM_170707.4)	Fig. 1G
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by luminescence: Negative	Supplementary Table 1A
Differentiation potential	e.g. Embryoid body formation OR Teratoma formation OR Scorecard OR Directed differentiation	Upregulated expression of specific genes for the three germ layers in embryoid bodies	Fig. 1F
List of recommended germ layer markers	Expression of these markers has to be demonstrated at mRNA (RT PCR)	Ectoderm: *KRT14, NCAM1, TH and GABBR2* Endoderm: *SOX7 and PDX1* Mesoderm: *CD31, ACTA2, SCL and CDH5*	Fig. 1F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 2
Reagents details.

	Antibodies used for immunocytochemistry/flow-cytometry			
	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
Pluripotency Markers (immunocytochemistry)	Mouse anti-TRA-1–60 (IgM)	1:100	Thermo Fisher Scientific Cat# A24868	RRID: AB_2651002
Differentiation Markers (immunocytochemistry)	Mouse Anti-Sarcomeric Alpha-Actin	1:100	Abcam Cat# ab9465	RRID:AB_307264
Differentiation Markers (immunocytochemistry)	Rabbit anti-Cardiac Troponin I	1:200	Abcam Cat# ab47003	RRID:AB_869982
Differentiation Markers (immunocytochemistry)	Mouse Anti-CD31	1:200	Agilent Cat# M0823	RRID: AB_2114471
Differentiation Markers (immunocytochemistry)	Rabbit Anti-TAGLN/Transgelin	1:200	Abcam Cat# ab14106	RRID:AB_443021)
Secondary antibodies	Alexa Fluor® 555 Donkey Anti-Rabbit	1:250	Thermo Fisher Scientific Cat# A24869	RRID: AB_2651006
Secondary antibodies	Alexa Fluor® 488 Goat Anti-Mouse IgG3	1:250	Thermo Fisher Scientific Cat# A24877	RRID: AB_2651008
Secondary antibodies	Alexa Fluor® 488 Donkey Anti-Rat	1:250	Thermo Fisher Scientific Cat# A24876	RRID: AB_2651007
Secondary antibodies	Alexa Fluor® 555 Goat Anti-Mouse IgM	1:250	Thermo Fisher Scientific Cat# A24871	RRID: AB_2651009
Secondary antibody	Alex Fluor® 488 Donkey anti-Mouse IgG	1:500	Thermo Fisher Scientific Cat# A-21202	RRID:AB_141607
Secondary antibody	Alex Fluor® 568 Donkey anti-Rabbit IgG	1:500	Thermo Fisher Scientific Cat# A10042	RRID: AB_2534017
Nuclear stain (immunocytochemistry)	Hoechst 33,342	2 μg/ml	Thermo Fisher Scientific Cat# H3570	N/A
Nuclear stain (immunocytochemistry)	DAPI (nuclear stain)	5 μg/ml	Thermo Fisher Scientific Cat# D3571	RRID: AB_2307445
	Primers			
	Target	**Size of band**	**Forward/Reverse primer (5′-3′)**	
House-Keeping Gene (qRT-PCR)	*GAPDH*	89 bp	CCACCCATGGCAAATTCC/ TCGCTCCTGGAAGATGGTG	
Pluripotency Markers (qRT-PCR)	*SOX2*	151 bp	GGGAAATGGGAGGGGTGCAAAAGAGG/ TTGCGTGAGTGTGGATGGGATTGGTG	
Pluripotency Markers (qRT-PCR)	*OCT3/4*	144 bp	GACAGGGGGAGGGGAGGAGCTAGG/ CTTCCCTCCAACCAGTTGCCCCAAAC	
Pluripotency Markers (qRT-PCR)	*NANOG*	116 bp	TGCAAGAACTCTCCAACATCCT/ ATTGCTATTCTTCGGCCAGTT	
Three germ layer markers (ectoderm) (qRT-PCR)	*KRT-14*	86 bp	CACCTCTCCTCCTCCCAGTT/ ATGACCTTGGTGCGGATTT	
Three germ layer markers (neuro-ectoderm) (qRT-PCR)	*NCAM-1*	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)	*GABRR-2*	106 bp	CTGTGCCTGCCAGAGTTTCA/ ACGGCCTTGACGTAGGAGA	
Three germ layer markers (endoderm) (qRT-PCR)	*SOX7*	112 bp	TGAACGCCTTCATGGTTTG/ AGCGCCTTCCACGACTTT	
Three germ layer markers (endoderm) (qRT-PCR)	*PDX1*	145 bp	AAGCTCACGCGTGGAAAG/ GGCCGTGAGATGTACTTGTTG	
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	
Sequencing	LMNA -Exon2	486 bp	AATGCAAGGATGCCCTCTCC/ ACTGTGGTAGATCCCATTGGC	

differentiate in all three germ layers was assessed by qRT-PCR after embryoid body (EB) formation, in which high expression levels of all genes related to ectoderm (*KRT-14, NCAM-1, TH, GABBR-2*), endoderm (*SOX7, PDX1*) and mesoderm (*CD31, ACTA2, SCL, CDH5*) were observed (Fig. 1F). The presence of the heterozygous *LMNA* mutation c.481G > A (NM_170707.4) was confirmed by Sanger sequencing (Fig. 1G). The genetic profile of this hiPSC line was compared with that of the parental PBMNCs, assessing 16 short tandem repeat (STR) markers (Table 1), and the result was identical to that of the donor cells.

Finally, the hiPSC line was differentiated using cardiomyogenic and endothelial differentiation protocols. In both cases, the obtained cells resulted positive for specific markers, as hiPSC-derived cardiomyocytes resulted positive for the sarcomeric markers α-actinin (green) and Troponin-I (red) (Fig. 1H) and hiPSC-derived endothelial cells resulted positive for CD31 (green) and negative for the muscle cell marker Transgelin (red) (Fig. 1I).

4. Materials and methods

4.1. Ethical statement

Blood was collected after patient signed informed consent. This study was approved by the Ethical Committee of Friuli Venezia Giulia Region, CERU N.O. 43/2009, Em02/2022.

4.2. Cell culture and reprogramming

PBMNCs were isolated from the donor, amplified and reprogrammed as previously described (Meraviglia et al., 2015).

After 3 days from the reprogramming cells were transferred on a MEF feeder layer coated plate (1.66×10^5/well) with a medium composed of KO-DMEM (Gibco) supplemented with 20% KO-Serum replacement (Gibco), 1 mM non-essential amino acids (Thermo Fisher Scientific), 1% penicillin/streptomycin (Biowest), 1% L-Glutamine (Biowest), 0,1% mM ß-mercaptoethanol (Thermo Fisher Scientific) and 10/ml basic fibroblast growth factor (Merck-Millipore) until the formation of the first colonies. The hiPSCs were cultured with this medium until passage 5 and split as clumps once a week using 1 mg/ml Collagenase IV (Thermo Fhiser Scientific). Finally, hiPSCs were adapted to feeder-free condition and plated on a Matrigel© (Corning) coated plate (0.083 mg/ml), cultured in StemMACS™ iPS-Brew XF (Miltenyi Biotec) supplemented with 1% penicillin/streptomycin (Biowest) and passaged twice a week using TrypLE™ (Thermo Fisher Scientific). To improve cell viability, the medium was supplemented with 10 μM Y-27632 (Miltenyi Biotec) when seeded after splitting. Cells were kept in an humified incubator at 37 °C with 5% CO_2 and 20% O_2.

4.3. Immunofluorescence staining

Cells were fixed with 4% PFA for 15 min. Immunostaining for pluripotency markers of undifferentiated hiPSCs was performed using the Pluripotent Stem Cell 4-Marker Immunocytochemistry Kit (Thermo Fisher Scientific) following manufacturer's instructions. Antibodies against the α-actinin, Troponin-I, CD31 and Transgelin, were used to test the cardiogenic and endothelial differentiation of hiPSCs.

4.4. qRT-PCR

Total RNA from PBMNCs, hiPSCs and EBs was extracted using TRIzol® reagent (Thermo Fisher Scientific). cDNA was obtained by retrotranscribing 1 μg of RNA using SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) following manufacturer's instructions.

For cDNA amplification All-in-One SYBR® Green qPCR Mix (GeneCopoeia) was used on the CFX96 Real-Time PCR Detection System (Biorad) using the following protocol: 95 °C × 10 min; 95 °C × 15 s – 60 °C × 45 s for 44 cycles; 95 °C × 1 min; melt curve 55 °C to 95 °C with an increment of 0.5 °C every 5 sec. The sequence of the primers used in this study are reported in Table 2.

4.5. Embryoid bodies

For the formation of embryoid bodies, hiPSCs in feeder-free conditions at passage 17 were detached and cultured in suspension in ultra-low attachment plates for 7 days in EBs 20% medium composed of KO-DMEM, 20% KO-serum, 1 mM NEAAs, 1% Penicillin/Streptomycin, 1% L-glutamine, 0,1% mM ß-mercaptoethanol. At day 7 EBs were transferred on a Matrigel© coated plate (0.083 mg/ml) in EBs 20% medium for a total of 25 days of differentiation. Cells were kept in an humified incubator at 37 °C with 5% CO_2 and 20% O_2.

4.6. Karyotype analysis

Karyotype analysis of EURACi015-A was performed at passage 23 using the Q-banding technique at 300–400 band resolution on 20 metaphase spreads.

4.7. STR analysis

Cell identity was assessed on genomic DNA from the hiPSC line and parental PBMNCs with the Applied BiosystemsTM AmpFLSTRTM IdentifierTM Plus PCR Amplification Kit, under ISO 17,025 accredited conditions (Eurofins Genomics). The kit includes the STR markers reported in Table 1.

4.8. Sequencing

Genomic DNA for sequencing and STR analysis was extracted from parental PBMNCs and reprogrammed hiPSCs using the DNeasy® Blood & Tissue Kit (Qiagen). The GoTaq® G2 Flexi DNA Polymerase kit (Promega) and sequencing primers listed in Table 2 were used for PCR reactions. The genomic region of interest was amplified with the 2720 thermal cycler (Applied Biosystem) using the following amplification parameters: 95 °C 2 min/95 °C 30 s; 61 °C 30 s; 72 °C 45 s for 30 cycles/ 72 °C 5 min; 4 °C 10 min. PCR products were purified by agarose gel extraction using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) and sent to Eurofins Genomic (Germany) for Sanger sequencing.

4.9. Mycoplasma test

Mycoplasma test was performed using MycoAlert™ *Mycoplasma* Detection Kit (Lonza), following manufacturer's instructions (Supplementary Table 1).

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alessandra Rossini and Serena Zacchigna reports financial support was provided by European Regional Development Fund.

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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.2023.103172.

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