Lab resource: Stem cell line

**Derivation of human induced pluripotent stem cell line EURACi004-A from skin fibroblasts of a patient with Arrhythmogenic Cardiomyopathy carrying the heterozygous PKP2 mutation c.2569_3018del50**

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**ABSTRACT**

Arrhythmogenic Cardiomyopathy (ACM) is an inherited cardiac disease characterized by arrhythmias and fibro-fatty replacement in the ventricular myocardium. Causative mutations are mainly reported in desmosomal genes, especially in plakophilin2 (PKP2). Here, using a virus-free reprogramming approach, we generated induced pluripotent stem cells (iPSCs) from skin fibroblasts of one ACM patient carrying the frameshift heterozygous PKP2 mutation c.2569_3018del50. The iPSC line (EURACi004-A) showed the typical morphology of pluripotent cells, possessed normal karyotype and exhibited pluripotency markers and trilineage differentiation potential, including cardiomyogenic capability. Thus, this line can represent a human in vitro model to study the molecular basis of ACM.

**Resource table**

| Unique stem cell line identifier | EURACi004-A |
|----------------------------------|-------------|
| Alternative name(s) of stem cell line | N/A |
| Institution | Institute for Biomedicine, Eurac Research, Bolzano, Italy |
| Contact information of distributor | Alessandra Rossini (alessandra.rossini@eurac.edu) |
| Type of cell line | iPSCs |
| Origin | Human |
| Additional origin info | Age: 34-year-old |
| Cell source | Skin fibroblasts |
| Clonality | Clonal |
| Method of reprogramming | Electroporation of episomal vectors (pCXLE hOCT3/4-shp53-F, pCXLE-hSK, and pCXLE-hUL) |
| Genetic modification | YES |
| Type of modification | Spontaneous mutation |
| Associated disease | Arrhythmogenic Cardiomyopathy |
| Gene/locus | Heterozygous PKP2 c.2569_3018del50 |
| Method of modification | N/A |
| Name of transgene or resistance | N/A |
| Inducible/constitutive system | N/A |
| Date archived/stock date | January 2015 |
| Cell line repository/bank | N/A |
| Ethical approval | Skin fibroblasts were collected after patient informed consent and after approval from Centro Cardiologico Monzino – IRCCS |

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Characterization and validation.

Table 1

| Classification                  | Test                          | Result                                                                 | Data                          |
|--------------------------------|-------------------------------|------------------------------------------------------------------------|-------------------------------|
| Morphology                     | Light microscopy              | Normal morphology and alkaline phosphate positivity                      | Fig. 1 panel A                |
|                                | Immunochemistry               | Expression of pluripotency markers: SSEA4, OCT4, TRA-1-60, SOX2        | Fig. 1 panel D                |
| Phenotype                      |                               | Fold change for pluripotency genes (RT-qPCR): SOX2 = 37.189 ± 430      | Fig. 1 panel A                |
|                                |                               | OCT4 = 13.0 ± 2                                                        | and Fig. 1 panel E (flow cytometry) |
|                                |                               | NANOG = 456 ± 64% of positive cells (flow cytometry analysis):         |                               |
|                                |                               | SOX2 98.1% ± 1.1%                                                      |                               |
|                                |                               | SSEA4 99% ± 0.9%                                                       | Fig. 1 panel I                |
| Genotype                       | Karyotype (Q-banding) Resolution: 300–400 bands | Normal karyotype: 46, XY                                                |                               |
| Identity                       | Microsatellite PCR (mPCR)     | Not performed                                                          | Available with the authors    |
|                                | STR analysis                  | Amelogenin (for gender identification), D3S1358, D1S1656, D6S1043,   |                               |
|                                |                               | D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01,  |                               |
|                                |                               | vWA, D21S11, D7S820, D5S818, TPOX, D8S1179, D12S391, D19S433 and FGA with 100% match |                               |
|                                |                               | Heterozygous PKP2 c.2569_3018del50                                     | Fig. 1 panel H                |
|                                |                               | N/A                                                                    | N/A                           |
|                                | Mycoplasma testing by luminescence: Negative | Mycoplasma testing by luminescence: Negative: Negative                     | Supplementary Fig. S1         |
| Mutation analysis (IF APPLICABLE) | Sequencing                 | Expression of genes of the three germ layers in embryoid bodies (SOX7 and AFP for endoderm, CD31, ACTA2, SCL and CDH5 for mesoderm; KRT14, NCAM1 and GABBR2 for ectoderm) and immunocytochemistry for spontaneous differentiation into cardiomyocytes | Fig. 1 panel F (gene expression analysis) and Fig. 1 panel G (immunocytochemistry for cardiac markers) |
| Microbiology and virology      | Southern Blot OR WGS         | N/A                                                                    |                               |
| Differentiation potential      | Embryoid body formation      | N/A                                                                    |                               |
| Donor screening (OPTIONAL)     | HIV 1 + 2 Hepatitis B, Hepatitis C | N/A                                                                    |                               |
| Genotype additional info (OPTIONAL) | Blood group genotyping | N/A                                                                    |                               |
|                                | HLA tissue typing             | N/A                                                                    |                               |

Resource utility

The established iPSC line (EURACi004-A) provides an unlimited source of in vitro human cardiomyocytes that can be a useful model to investigate the molecular basis of arrhythmia generation in ACM. This might help to identify pharmacological approaches to ameliorate the arrhythmogenic and/or metabolic phenotypes associated with ACM.

Resource details

Arrhythmogenic Cardiomyopathy (ACM) is a cardiac genetic disorder characterized by potentially lethal ventricular arrhythmias, progressive loss of cardiomyocytes and fibro-fatty replacement in the myocardium, mainly in the right ventricle. The disease usually has an autosomal dominant transmission with variable penetrance and expressivity. Most mutations have been found in genes encoding desmosomal proteins and PKP2 is the most common causal gene (Awad et al., 2006). ACM is a complex disease and the associated pathogenic mechanisms are still unclear. Given the difficulty to obtain isolated human cardiomyocytes for in vitro studies, patient-specific iPSCs constitute an unlimited source of human cardiomyocytes for disease modelling, thus allowing the elucidation of ACM pathophysiology.

Here, we generated iPSCs from skin fibroblasts of a 34 years old male carrying a heterozygous frameshift mutation in PKP2 gene (c.2569_3018del50), leading to a premature termination on the PKP2 protein, already described as associated with ACM (Antoniades et al., 2006). Three different iPSC clones were generated from this patient and the EURACi004-A iPSC clone reported in this work was randomly selected for the characterization reported in Table 1. In detail, skin fibroblasts were reprogrammed into iPSCs using electroporation of episomal plasmids carrying OCT3/4, SOX2, KLF4, and L-MYC (Meraviglia et al., 2016). The selected EURACi004-A iPSC was amplified on mouse embryonic fibroblasts (MEFs). iPSC colonies showed a typical human embryonic-stem cell morphology and displayed high positivity for alkaline phosphatase (Fig. 1A). Undifferentiated iPSCs expressed endogenous pluripotency genes (i.e. SOX2, OCT4, NANOG) at higher levels compared to parental skin fibroblasts (Fig. 1B).

Of note, EURACi004-A iPSC clone did not show the expression of episomal transgenes, evaluated by performing qPCR using primers for the episomal specific EBNA-1 gene, thus confirming successful activation of endogenous pluripotency genes without genome integration of exogenous plasmids (Fig. 1C).

The expression of transcription factors OCT4, SOX2 and surface markers SSEA4, TRA-1-60 associated with pluripotency was evaluated by immunofluorescence analysis (Fig. 1D), while the percentage of positive cells for SOX2 (98%) and SSEA4 (99%) was assessed by flow cytometry (Fig. 1E). The potential to differentiate into three germ lineages was evaluated after spontaneous differentiation through embryoid body (EB) formation by qRT-PCR on endodermal (SOX7 and AFP, green), mesodermal (CD31, ACTA2, SCL and CDH5, red) and ectodermal (KRT14, NCAM1 and GABBR2, blue) genes (Fig. 1F). Cardiomyogenic differentiation occurred spontaneously through embryoid body (EB) formation, as evident by the clear striated pattern of α-Antinin and Troponin I (Fig. 1G). As indicated in Fig. 1H, Sanger sequencing confirmed the presence of the PKP2 mutation and EURACi004-A iPSC line showed a normal karyotype at passage 33(Fig. 1I). STR analysis indicated that the newly created EURACi004-A iPSC clone yielded a 100% match with the skin fibroblast counterpart, confirming that they are correctly derived from the donor (Table 1, available with the authors). Finally, iPSCs resulted negative for mycoplasma contamination (Supplementary Fig. S1).
Materials and methods

Cell culture and reprogramming

Skin fibroblasts were isolated from skin biopsy and amplified as previously described (Meraviglia et al., 2016). iPSCs from skin fibroblasts were generated using the episomal vectors pCXLE-hOCT3/4-shp53-F, pCXLE-hSK, and pCXLE-hUL (Addgene). Specifically, 7.5 × 10^5 skin fibroblasts were electroporated with 1 μg of each episomal vector using the Neon System (Thermo Fisher Scientific) at the program: 1650 V, 10 msec, 3 pulses and plated onto a plastic tissue culture dish for seven days in standard culture medium composed of Iscove’s Modified Dulbecco’s Medium (IMDM) (Thermo Fisher Scientific), 20% FBS Defined (Hyclone), 1% Penicillin/Streptomycin, 1% L-Glutamine (all from Thermo Fisher Scientific). Then, transfected fibroblasts were trypsinized, plated on 0.1% gelatin-coated plate with irradiated MEF feeder layer 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). Then, transfected fibroblasts were trypsinized, plated on 0.1% gelatin-coated plate with irradiated MEF feeder layer 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). Then, transfected fibroblasts were trypsinized, plated on 0.1% gelatin-coated plate with irradiated MEF feeder layer 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). Then, transfected fibroblasts were trypsinized, plated on 0.1% gelatin-coated plate with irradiated MEF feeder layer 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). Then, transfected fibroblasts were trypsinized, plated on 0.1% gelatin-coated plate with irradiated MEF feeder layer 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). Then, transfected fibroblasts were trypsinized, plated on 0.1% gelatin-coated plate with irradiated MEF feeder layer 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). Then, transfected fibroblasts were trypsinized, plated on 0.1% gelatin-coated plate with irradiated MEF feeder layer 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). Then, transfected fibroblasts were trypsinized, plated on 0.1% gelatin-coated plate with irradiated MEF feeder layer and cultured in stem cell medium containing knockout DMEM, 20% KO-Serum Replacement (KOSR), 1 mM NEAAs, 1% Penicillin/Streptomycin, 1% L-Glutamin

qRT-PCR

Total RNA from skin fibroblasts, iPSCs and EBs was extracted using TRIzol® Reagent. Reverse transcription of RNA (1 μg) was performed 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 for cDNA amplification on CFX96 Real-Time PCR Detection System (BioRad). Table 2 reports primer sequences.

Immunofluorescence staining

Pluripotent Stem Cell 4-Marker Immunocytochemistry Kit (Thermo Fisher Scientific) was used for the detection of pluripotency markers on undifferentiated iPSCs, according to manufacturer’s instructions (Table 2). Cardiomyocytes at 30–35 days of differentiation were fixed in PFA 4% for 10 min and permeabilized with 0.2% of Triton-100× at room temperature for 7 min. Blocking buffer (5% goat serum) was added for 1 h at room temperature. Primary antibodies were incubated overnight at 4 °C, while secondary antibodies were incubated for 1 h at 37 °C (Table 2). Nuclei were counterstained with DAPI. All the images have been acquired using Leica SP8-X confocal microscope.

Flow cytometry

SOX2 and SSEA4 expression in iPSCs adapted to feeder-free condition was analyzed by flow cytometry using Multi-Color Flow Cytometry Kit (R&D System) following manufacturer’s instruction. Samples were
analyzed using the S3e Cell Sorter (BioRad). Flow cytometry data analysis was performed using Flowing Software 2.5.1.

**Embryoid body formation**

iPSCs were differentiated as embryoid bodies (EBs) by detaching colonies and growing them in ultra-low attachment plates for 7 days in 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 days. Differentiation, spontaneous beating clusters, indicating iPSC spontaneous cardiomyogenic ability, were manually microdissected, dissociated at single cell level using TrypLE and planted onto gelatin-coated plates in EB 2% medium (same EB medium except for supplementation with only 2% FBS) for additional 5–10 days, in order to avoid fibroblast over-growth.

**Karyotyping**

Karyotype evaluation was assessed on iPSC adapted to feeder-free condition after 33 in vitro passages by cytogenetic Q-banding analysis as previously described (Meraviglia et al., 2015). Specifically, 20 metaphases were analyzed and all metaphases showed a normal karyogram.

**Sequence and STR analysis**

Genomic DNA used for PKP2 sequencing and STR analysis was isolated using DNeasy Blood and Tissue kit (Qiagen), following manufacturer’s instructions. PKP2 mutation analysis was performed on a PCR product obtained by genomic DNA amplification using Kapa High Fidelity DNA Polymerase (Kapa Biosystems), following manufacturer’s instructions (see primers listed in Table 2, mutation analysis sequencing). The PCR reaction has been performed on Mastercycler® pro S instrument (Eppendorf) using the following conditions: 95°C 5 min/98°C 20 s; 60°C 20 s; 72°C 30 s for 35 cycles, 72°C 1 min 30 s; 5°C 10 min; 16°C ∞. The amplification product was purified using Agencourt AMPure PCR purification system (Beckman Coulter) and PCR product size was inspected using agarose-gel electrophoresis (agarose gel 1%). Then, PKP2 mutation was confirmed on iPSCs and their skin fibroblast counterpart on the amplification product by Sanger sequencing performed by Eurofins Genomics (Germany), according to ANSI/ATCC standard ASN-0002 (Table 1), using a combined set of forward and reverse primers (Table 2, see mutation analysis sequencing).
Specifically, cell identity was analyzed on genomic DNA, evaluating 21 independent loci by PCR-single-locus-technology (Promega, PowerPlex 21 PCR Kit).

### Mycoplasma test

Mycoplasma test

MycoAlert™ Mycoplasma Detection Kit (Lonza) was used to test mycoplasma contamination, following manufacturer's instructions (Table 2).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.09.003.

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