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Lab resource: Multiple cell lines

Generation of human induced pluripotent stem cells (EURACi001-A, EURACi002-A, EURACi003-A) from peripheral blood mononuclear cells of three patients carrying mutations in the CAV3 gene

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ABSTRACT

Caveolinopathies are a heterogeneous family of genetic pathologies arising from alterations of the caveolin-3 gene (*CAV3*), encoding for the isoform specifically constituting muscle caveolae. Here, by reprogramming peripheral blood mononuclear cells, we report the generation of induced pluripotent stem cells (iPSCs) from three patients carrying the ΔYTT deletion, T78K and W101C missense mutations in caveolin-3. iPSCs displayed normal karyotypes and all the features of pluripotent stem cells in terms of morphology, specific marker expression and ability to differentiate *in vitro* into the three germ layers. These lines thus represent a human cellular model to study the molecular basis of caveolinopathies.

Resource Table

Unique stem cell lines identifier	EURACi001-A
	EURACi002-A
	EURACi003-A
Alternative names of stem cell lines	B2CAV3 (EURACi001-A) L1CAV3 (EURACi002-A) N1CAV3 (EURACi003-A)
Institution	Institute for Biomedicine, Eurac Research
Contact information of distributor	Alessandra Rossini (alessandra.rossini@eurac.edu)
Type of cell lines	iPSCs
Origin	Human
Cell source	Peripheral blood mononuclear cells (PBMCs)

(*continued*)

Unique stem cell lines identifier	EURACi001-A
	EURACi002-A
	EURACi003-A
Method of reprogramming	Electroporation of episomal vectors (pCXLE hOCT3/4-shp53-F, pCXLE-hSK, and pCXLE-hUL)
Multiline rationale	Non-isogenic cell lines obtained from patients with mutations in the same gene (CAV3)
Gene modification	NO
Type of modification	Spontaneous mutations
Associated disease	Caveolinopathies
Gene/locus	Heterozygous CAV3 c.Δ184–192 (EURACi001-A) Heterozygous CAV3 c.303 TGG > TGC (EURACi002-A) Heterozygous CAV3 c.233 ACG > AAG (EURACi003-A)
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	January 2016 (EURACi001-A) September 2016 (EURACi002-A)

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(*continued*)

Unique stem cell lines identifier	EURACi001-A
	EURACi002-A
	EURACi003-A
	May 2016 (EURACi003-A)
Cell line repository/bank	N/A
Ethical approval	Peripheral blood was collected from patients after signing the informed consent provided by Cell Line and DNA Biobank from Patients Affected by Genetic Diseases, member of the Telethon Network of Genetic Biobanks, Istituto G. Gaslini, Genoa, Italy. The generation and use of iPSCs was reviewed and approved by Ethical Committee at Universita' degli Studi di Milano (03.06.15, number 29/15).

Resource utility

The established iPSC lines (EURACi001-A, EURACi002-A, EURACi003-A) provide a resource to generate *in vitro* human cardiomyocytes and myoblast/myotubes in order to understand the molecular and functional mechanisms leading to altered cellular excitability typical of these pathological *CAV3* mutations and to evaluate a pharmacological approach to eventually correct such alterations.

Resource details

The term caveolinopathies refers to a group of primary myopathies resulting from alteration of the proteins named caveolin-3, the main constituent of muscle caveolae. These genetic disorders are mainly inherited through an autosomal dominant pattern. The plasma membrane of cardiomyocytes is rich in caveolae and caveolins play multiple roles in the physiology of cardiovascular system (Panneerselvam et al., 2012). Beyond its role in maintaining the structural integrity of caveolae, caveolin-3 is known to interact with several ion channels in the heart, modifying their functional properties and expression levels (Barbuti et al., 2012; Campostrini et al., 2017). In order to elucidate in detail the role of *CAV3* mutations in skeletal muscle and cardiac diseases, the availability of large quantities of human skeletal muscle and cardiac myocytes is a major requirement. Since iPSC can be differentiated in both these cell types, they provide an unlimited source of human cells otherwise unavailable. We generated iPSCs from three patients carrying different heterozygous mutations in CAV3 genes (Table 1): one patient who carries a deletion c.Δ184-192 (ΔYTT), one with c.303 TGG > TGC (W101C) mutation and another patient showing c.233 ACG > AAG (T78K) mutation. At least four iPSC clones were generated from each patient and the iPSC clones (EURACi001-A, EURACi002-A, EURACi003-A) reported in this work were randomly selected and successfully characterized. Specifically, iPSCs were obtained from peripheral blood mononuclear cells (PBMCs) using electroporation of episomal plasmids carrying OCT3/4, SOX2, KLF4, and L-MYC (Meraviglia et al., 2015). iPSC colonies showed a typical human embryonic-stem cell morphology and were positive for alkaline phosphatase staining (Supplementary Fig. 1A). qRT-PCR analysis revealed that all iPSC lines expressed endogenous pluripotency genes (SOX2, OCT3, NANOG) at higher levels compared to their PBMCs counterpart (Supplementary Fig. 1B). Immunofluorescence analysis showed the expression of the pluripotency markers OCT4, SOX2 at nuclear level and SSEA4, TRA-1-60 as surface proteins (Fig. 1A). Flow cytometry allowed to quantify the percentage of cell positive for SSEA4 (≥97.8%) and SOX2 (≥79.8) in all the three iPSC lines (Fig. 1B). The ability of iPSC lines to differentiate into cells of the three germ layers following embryoid body (EB) formation was assessed by qPCR 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. 1C). Furthermore, direct cardiomyogenic differentiation of all iPSC lines led to the formation of cardiomyocytes, as indicated by immunofluorescence staining of specific cardiac marker (α-Actinin), which displayed a clear sarcomeric pattern (Fig. 1D). Also, cytogenetic analysis was performed on all iPSC lines, showing a normal karyotype (Supplementary Fig. 1C) and genomic DNA sequencing confirmed the presence of all the three mutations in CAV3 gene (Supplementary Fig. 1D). Then, STR analysis indicated that iPSCs and their PBMC counterparts yielded a match ≥98%, confirming that they are correctly derived from the donor (Table 2, data not shown). Finally, PCR results indicated that all the iPSC lines were negative for mycoplasma contamination (Supplementary Fig. 1E).

1. Materials and methods

1.1. Cell culture and reprogramming

PBMCs obtained from venous peripheral blood were amplified in suspension as previously described (Meraviglia et al., 2015). 5×10^6 PBMCs were reprogrammed by electroporation of 1 μg of each episomal vector (pCXLE-hOCT3/4-shp53-F, pCXLE-hSK, and pCXLE-hUL from Addgene) using Neon System (Thermo Fisher Scientific) following the program: 1650 V, 10 ms, 3 pulses. Three days after electroporation, transfected PBMCs were plated on a feeder layer of irradiated mouse embryonic fibroblasts (MEFs) and cultured in iPSC medium containing knockout 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) (Meraviglia et al., 2015). IPSCs maintained on MEFs were expanded by enzymatic dissociation using 1 mg/ml Collagenase IV (Thermo Fisher Scientific). IPSCs adapted to feeder-free condition were cultured in TeSR-E8 medium (Stem Cell Technologies) on Matrigel matrix (Corning) and propagated using PBS-EDTA 0.5 mM.

1.2. iPSC differentiation

For differentiation into the three germ layer, iPSCs on MEFs were detached and grown in ultra-low attachment plates for 7 days to form EBs in 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 for further 10–15 days of differentiation.

Cardiomyogenic differentiation was performed on iPSC cultured in feeder free condition, plating 20,000 cells/cm^2 on 24-well plate coated with Matrigel and using PSC Cardiomyocyte Differentiation Kit (Thermo Fisher Scientific), according to manufacturer's instructions.

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
EURACi001-A	EURACi001-A	Female	36	Caucasian	Het c.Δ184-192	Limb-girdle muscular dystrophy (LGMD-1C)
EURACi002-A	EURACi002-A	Female	22	Caucasian	Het c.303 TGG > TGC	Isolated Hyper-CKEmia
EURACi003-A	EURACi003-A	Female	43	Caucasian	Het c.233 ACG > AAG	Hyper-CKemia and Rippling Muscle Disease

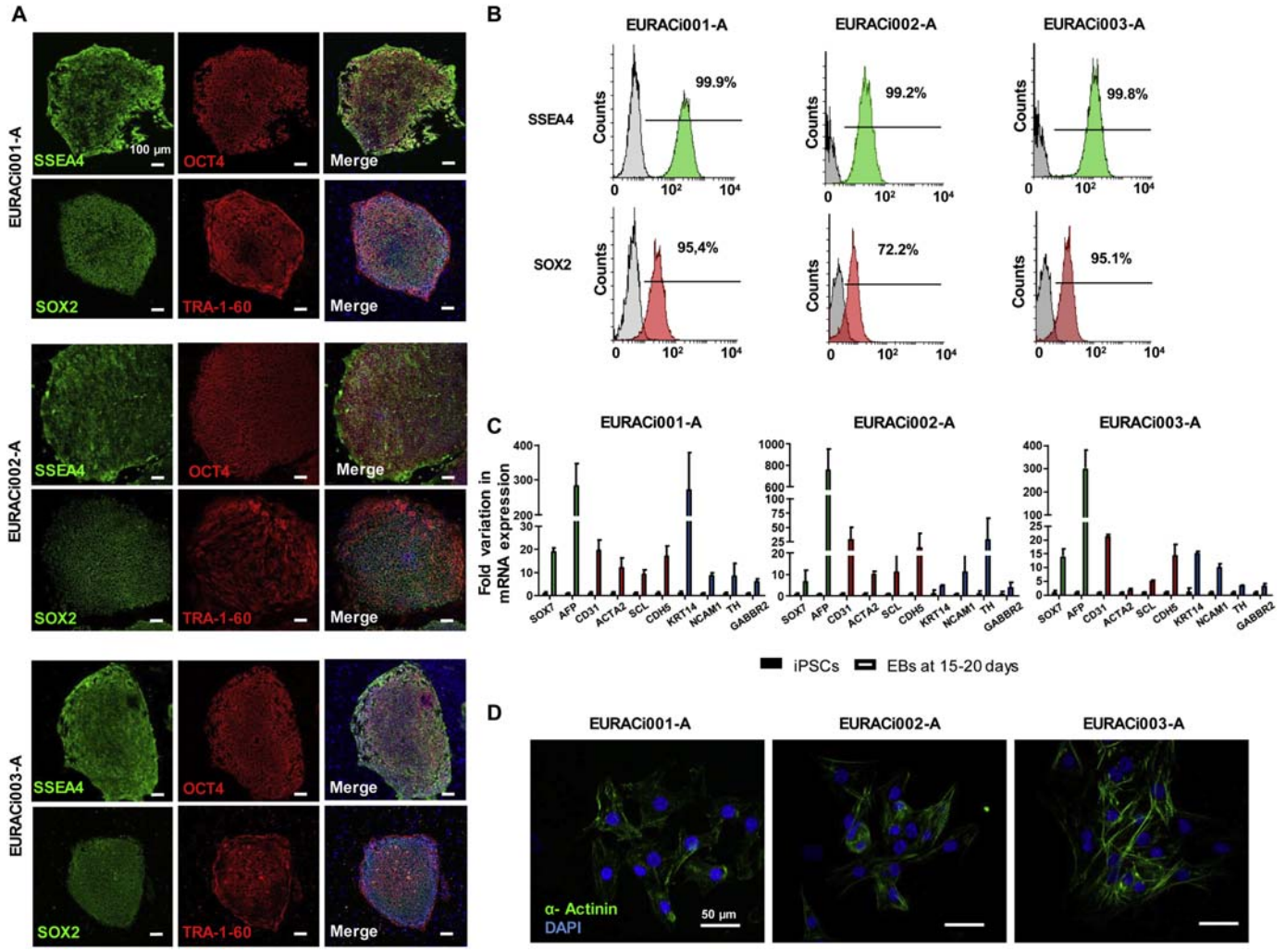

Fig. 1. Generation and characterization of three iPSC lines EURACi001-A, EURACi002-A and EURACi003-A. (A) Immunofluorescence staining for pluripotency markers SSEA4, OCT4, SOX2 and TRA-1-60; scale bar 100 µm. (B) Flow citometry analysis of pluripotency markers SSEA4 ad SOX2. (C) Gene expression analysis of three germ layer markers after iPSC differentiation via embryoid body formation. (D) Representative immunofluorescence images for sarcomeric protein α-Actinin after direct cardiomyogenic differentiation; scale bar 50 µm.

1.3. Immunofluorescence staining

Pluripotency markers were detected using Pluripotent Stem Cell 4-Marker Immunocytochemistry Kit (Thermo Fisher Scientific) as indicated by manufacturer's instruction (Table 3).

After 35 days of differentiation, cardiomyocytes were fixed in PFA 4% for 10 min at room temperature. Blocking of unspecific sites was achieved by incubation with 3% donkey serum in the presence of 0.3% Triton-X100 for 1 h at room temperature. Primary antibodies were incubated overnight at 4 °C, while secondary antibodies were incubated 45 min at room temperature (Table 3). Nuclei were stained with DAPI (Sigma-Aldrich).

1.4. Flow cytometry

Flow Cytometry analysis was performed using Multi-Color Flow Cytometry Kit (R&D System) following manufacturer's instruction. Samples were analyzed using FACSAriaII™ flow cytometry (BD Bioscience).

1.5. qRT-PCR

Total RNA from PBMCs, iPSCs and EBs was extracted using TRIzol® Reagent and 1 µg of RNA was reverse transcribed using SuperScript VILO cDNA Synthesis Kit following manufacturer's instruction (all from Thermo Fisher Scientific). cDNA was amplified using All-in-One SYBR® Green qPCR Mix (GeneCopoeia) on CFX96 Real-Time PCR Detection System (BioRad). Primers sequences were reported in Table 3.

1.6. Karyotiping

Cytogenetic Q-banding analysis was performed as previously described (Meraviglia et al., 2015).

1.7. Sequencing and STR analysis

Genomic DNA from both iPSCs and corresponding PBMCs was sent to Eurofins Genomics (Germany) for analysis, according to ANSI/ATCC standard ASN-0002 (Table 2). In particular, 21 independent loci were investigated by PCR-single-locus-technology (Promega, PowerPlex 21 PCR Kit).

1.8. Mycoplasma test

The mycoplasma contamination test was carried out by PCR using primers able to recognize most of the Mycoplasma species (Table 3).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2017.12.012.

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Light miscroscopy	Normal morphology and alkaline phosphatase positivity	Supplementary Fig. 1A
Phenotype	Immunocytochemisty	Expression of pluripotency markers: SSEA4, OCT4, TRA-1-60, SOX2	Fig. 1A
	Flow cytometry	EURACi001-A positivity to: SSEA4 99.9 ± 0.05%; SOX2 = 96.4 ± 0.94% EURACi002-A positivity to: SSEA4 99.4% ± 0.19%; SOX2 79.8% ± 7.56% EURACi003-A positivity to: SSEA4 97.8% ± 2.07%; SOX2 88.0% ± 7.06%	Fig. 1B
Genotype	Karyotype (Q-banding) Resolution: 300–400 bands	Normal karyotype: 46, XX for all the three iPSC lines	Supplementary Fig. 1C
Identity	Microsatellite PCR (mPCR)	Not performed	
	STR analysis	21 markers tested: 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 (Promega PowerPlex 21 Kit) with match ≥98%	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	c.Δ184–192 (EURACi001-A) c.303 TGG > TGC (EURACi002-A) c.233 ACG > AAG (EURACi003-A)	Supplementary Fig. 1D
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR: Negative	Supplementary Fig. 1E
Differentiation potential	Embryoid body formation AND Direct differentiation	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, TH and GABBR2 for ectoderm) and directed differentiation into cardiomyocytes	Fig. 1C (gene expression analysis) and Fig. 1D (immunocytochemistry for cardiac marker)
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 3
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry

	Antibody	Dilution	Company Cat # and 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
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
Secondary antibodies (immunocytochemistry)	Alexa Fluor® 555 Goat Anti-Mouse IgM	1:250	Thermo Fisher Scientific Cat# A24871, RRID:AB_2651009
Pluripotency markers (flow cytometry)	PE-SOX2 Mouse IgG2A	1:20	R&D System Cat# IC2018P, RRID:AB_357273
Pluripotency markers (flow cytometry)	CFS-SSEA-4 Mouse IgG3	1:20	R&D System Cat# FAB1435F, RRID:AB_952015
Pluripotency markers (flow cytometry)	PE Isotype control- mouse IgG2A	1:20	R&D System Cat# IC003P, RRID:AB_357245
Pluripotency markers (flow cytometry)	CFS Isotype control- Mouse IgG3	1:20	R&D System Cat# IC007F, RRID:AB_952037
Cardiomyocyte markers (immunocytochemistry)	Mouse anti-Sarcomeric actin	1:400	Sigma Aldrich Cat# A7732, RRID:AB_2221571
Secondary antibodies (immunocytochemistry)	Alexa Fluor® 488 Goat Anti-Mouse IgG	1:600	Thermo Fisher Scientific Cat# A21202, RRID:AB_141607

Primers

	Target	Forward/reverse primer (5′–3′)
Pluripotency Markers (qRT-PCR)	SOX2	GGGAAATGGGAGGGGTGCAAAAGAGG/TTGCGTGAGTGTGGATGGGATTGGTG
Pluripotency Markers (qRT-PCR)	OCT4	GACAGGGGGAGGGGAGGAGCTAGG/CTTCCCTCCAACCAGTTGCCCCAAAC
Pluripotency Markers (qRT-PCR)	NANOG	TGCAAGAACTCTCCAACATCCT/ATTGCTATTCTTCGGCCAGTT
Three germ layer markers (endoderm) (qRT-PCR)	SOX7	TGAACGCCTTCATGGTTTG/AGCGCCTTCCACGACTTT
Three germ layer markers (endoderm) (qRT-PCR)	AFP	GTGCCAAGCTCAGGGTGTAG/CAGCCTCAAGTTGTTCCTCTG
Three germ layer markers (mesoderm) (qRT-PCR)	CD31	ATGCCGTGGAAAGCAGATAC/CTGTTCTTCTCGGAACATGGA
Three germ layer markers (mesoderm) (qRT-PCR)	ACTA2	GTGATCACCATCGGAAATGAA/TCATGATGCTGTTGTAGGTGGT
Three germ layer markers (mesoderm) (qRT-PCR)	SCL	CCAACAATCGAGTGAAGAGGA/CCGGCTGTTGGTGAAGATAC
Three germ layer markers (mesoderm) (qRT-PCR)	CDH5	GAGCATCCAGGCAGTGGTAG/CAGGAAGATGAGCAGGGTGA
Three germ layer markers (ectoderm) (qRT-PCR)	KRT14	CACCTCTCCTCCTCCCAGTT/ATGACCTTGGTGCGGATTT
Three germ layer markers (neuro-ectoderm) (qRT-PCR)	NCAM1	CAGATGGGAGAGGATGGAAA/CAGACGGGAGCCTGATCTCT
Three germ layer markers (neuro-ectoderm) (qRT-PCR)	TH	TGTACTGGTTCACGGTGGAGT/TCTCAGGCTCCTCAGACAGG
Three germ layer markers (neuro-ectoderm) (qRT-PCR)	GABRR2	CTGTGCCTGCCAGAGTTTCA/ACGGCCTTGACGTAGGAGA
House-Keeping Gene (qRT-PCR)	GAPDH	CCACCCATGGCAAATTCC/TCGCTCCTGGAAGATGGTG
Mutation analysis (sequencing)	CAV3	TGTGGGCACCTACAGCTTTGAC/CACCTGGCTTTAGACCTCCTTC
Mycoplasma detection		TGCACCATCTGTCACTCTGTTAACCTC/ACTCCTACGGGAGGCAGCAGTA

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