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Lab Resource: Multiple Cell Lines

Generation of two iPSC lines (FAMRCi007-A and FAMRCi007-B) from patient with Emery–Dreifuss muscular dystrophy and heart rhythm abnormalities carrying genetic variant *LMNA* p.Arg249Gln

Kseniya Perepelina[a,b,c], Aleksandra Kostina[c], Polina Klauzen[b,c], Aleksandr Khudiakov[a], Martina Rabino[g], Silvia Crasto[g], Anna Zlotina[a,b], Yulia Fomicheva[a], Alexey Sergushichev[d], Mari Oganesian[a], Alexander Dmitriev[b,e], Anna Kostareva[a,b,f], Elisa Di Pasquale[g,h,*], Anna Malashicheva[a,b,c,*]

[a] *Almazov National Medical Research Centre, Saint-Petersburg, Russia*
[b] *Saint Petersburg State University, Saint-Petersburg, Russia*
[c] *Institute of Cytology RAS, Saint-Petersburg, Russia*
[d] *ITMO University, Saint-Petersburg, Russia*
[e] *Federal State Budgetary Scientific Institution "Institute of Experimental Medicine", Saint-Petersburg, Russia*
[f] *Department of Women's and Children's Health, Karolinska Institute, Stockholm, Sweden*
[g] *Humanitas Clinical and Research Center, Rozzano, (MI), Italy*
[h] *Institute of Genetic and Biomedical Research (IRGB), UOS of Milan, National Research Council (CNR) of Italy, Italy*

ABSTRACT

Human iPSC lines were generated from peripheral blood mononuclear cells of patient carrying *LMNA* mutation associated with Emery–Dreifuss muscular dystrophy accompanied by atrioventricular block and paroxysmal atrial fibrillation. Reprogramming factors *OCT4*, *KLF4*, *SOX2*, *CMYC* were delivered using Sendai virus transduction. iPSCs were characterized in order to prove the pluripotency markers expression, normal karyotype, ability to differentiate into three embryonic germ layers. Generated iPSC lines would be useful model to investigate disease development associated with genetic variants in *LMNA* gene.

1. Resource Table

Unique stem cell lines identifier	FAMRCi007-A, FAMRCi007-B
Alternative names of stem cell lines	NA
Institution	Almazov National Medical Research Centre
Contact information of distributor	Anna Malashicheva amalashicheva@gmail.com and Elisa Di Pasquale, elisadipa@gmail.com
Type of cell lines	iPSC
Origin	Human
Cell Source	Peripheral Blood Mononuclear Cells
Clonality	Clonal
Method of reprogramming	Sendai virus
Multiline rationale	same disease isogenic cell lines
Gene modification	YES
Type of modification	Hereditary
Associated disease	Emery-Dreifuss muscular dystrophy, atrioventricular block, paroxysmal atrial fibrillation
Gene/locus	*LMNA* NM_170707.3:c.746G > A, NP_733821.1:p.Arg249Gln, (chr1 156104702G > A, rs59332535)
Method of modification	NA
Name of transgene or resistance	NA
Inducible/constitutive system	NA
Date archived/stock date	29th October 2019
Cell line repository/bank	https://hpscreg.eu/cell-line/FAMRCi007-A https://hpscreg.eu/cell-line/FAMRCi007-B
Ethical approval	The local ethical committee of Almazov National Medical Research Centre approval obtained. Patient informed consent obtained

2. Resource utility

Generated iPSC cell lines FAMRCi007-A and FAMRCi007-B are applicable for disease modelling of laminopathies linked to muscle and cardiac pathologies (Tables 1 and 2).

* Corresponding authors.
E-mail addresses: elisadipa@gmail.com (E. Di Pasquale), amalashicheva@gmail.com (A. Malashicheva).

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Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
FAMRCi007-A	FAMRCi007-A	Male	18	Caucasian	*LMNA* NM_170707.3:c.746G > A, NP_733821.1:p.Arg249Gln, (chr1 1561047020G > A, rs59332535)	Emery-Dreifuss muscular dystrophy, atrioventricular block, paroxysmal atrial fibrillation
FAMRCi007-B	FAMRCi007-B	Male	18	Caucasian	*LMNA* NM_170707.3:c.746G > A, NP_733821.1:p.Arg249Gln, (chr1 1561047020G > A, rs59332535)	Emery-Dreifuss muscular dystrophy, atrioventricular block, paroxysmal atrial fibrillation

3. Resource details

LMNA gene encodes nuclear lamins A type. Mutations in this gene cause a broad range of tissue-specific diseases called laminopathies (Burke and Stewart, 2013). Here we have reprogrammed PBMCs from an 18-year-old male patient carrying LMNA p.Arg249Gln genetic variant recently identified as pathogenic (Di Barletta et al., 2000). Patient suffered from Emery-Dreifuss muscular dystrophy accompanied by atrioventricular block and paroxysmal atrial fibrillation. We have obtained and characterized two iPSC lines (FAMRCi007-A and FAMRCi007-B). Patient's PBMCs were reprogrammed using CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific) according to the manufacturer's recommendation. iPSCs colonies were maintained on mitomycin C (Serva) treated mouse embryonic fibroblasts cells (MEF) and then adapted to feeder-free conditions for further characterization. Generated iPSC lines showed typical embryonic stem cell-like morphology how it is shown with the phase-contrast image (Fig. 1A). Immunofluorescent staining was performed to prove the expression of pluripotent markers (Fig. 1B) using the antibodies against human OCT4, NANOG and SSEA4. Obtained FAMRCi007-A and FAMRCi007-B lines were positive for alkaline phosphatase staining (Fig. 1C). QPCR analysis revealed that FAMRCi007-A and FAMRCi007-B lines expressed pluripotent markers *OCT4, NANOG* and *SOX2* (Fig. 1D). Elimination of reprogramming factors in the FAMRCi007-A and FAMRCi007-B was confirmed by RT-PCR (Fig. 1E). Patient-specific LMNA p.Arg249Gln genetic variant in iPSC lines was confirmed by Sanger sequencing (Fig. 1F). Both iPSC lines had a normal 46, XY karyotype. Comparative genomic hybridization array (CGH array) analysis does not reveal translocations or inversions, alterations in chromosome structure, mosaicism or polyploidy (Fig. 1G). Flow cytometry quantitative analysis revealed high expression of human iPSC-specific surface marker SEEA4 in FAMRCi007-A and FAMRCi007-B lines (Fig. 1H). In vitro trilineage differentiation was performed to confirm pluripotency of the established iPSC lines (Fig. 1I). The data of STR analysis proved the genetic identity between the established iPSC lines and the patient's PBMCs (Supplementary file). FAMRCi007-A and FAMRCi007-B lines were negative for mycoplasma contamination (Supplementary file). To sum up, generated iPSC lines carrying LMNA p.Arg249Gln genetic variant enables *in vitro* functional studies for investigation of laminopathies development.

4. Materials and methods

4.1. Ethic statement

The study was performed according to Declaration of Helsinki and with the approval of local ethical committee of Almazov National Medical Research Centre (№ 13/19.06.2014). Written informed consent was obtained prior to patient enrollment.

4.2. Generation and cultivation of iPSCs

Peripheral blood mononuclear cells (PBMCs) were isolated from anticoagulated blood using a density gradient centrifugation on Ficoll-Paque PLUS density gradient media (GE Healthcare). Obtained PBMCs were incubated in RPMI medium supplemented with 10% FBS, 50 U/ml IL-2 and T-Activator Purified NA/LE Mouse Anti-Human CD3/CD282 (BD Biosciences) for 4 days. Activated T cells were further reprogrammed using CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. iPSCs colonies were maintained on mitomycin C (Serva) treated mouse embryonic fibroblasts cells (MEF) in medium containing KO-DMEM supplemented with 20% KOSR, 2 mM glutamine, 0.1 mM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin (Thermo Fisher Scientific), 100 μM β-mercaptoethanol (Sigma), 20 ng/ml bFGF (Peprotech), B-27 Supplement without Vitamin A (Thermo Fisher

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Visual record of the line: normal	Fig. 1 panel A
Phenotype	Qualitative analysis (Immunocytochemistry)	OCT4, NANOG, SSEA4	Fig. 1 panel B
	Qualitative analysis (Alkaline Phosphatase staining)	Positive	Fig. 1 panel C
	Qualitative analysis (Flow cytometry)	SSEA4: 99.8% (FAMRCi007-A) SSEA4: 99.8% (FAMRCi007-B)	Fig. 1 panel H
Genotype	Karyotype (CGH-array)	46, XY Resolution 82 Kb	Fig. 1 panel G
Identity	Microsatellite PCR (mPCR) OR	NA	NA
	STR analysis	11 STR loci were tested, all matched	Submitted as Supplementary file
Mutation analysis (IF APPLICABLE)	Sequencing	Heterozygous mutation	Fig. 1 panel F
	Southern Blot OR WGS	NA	NA
Microbiology and virology	Mycoplasma	Mycoplasma testing by RT-PCR, negative	Submitted as Supplementary file
Differentiation potential	Directed differentiation	Proofs of three germ layers formation are Expression of proteins (ectoderm: βIII tubulin; mesoderm: Brachyury; endoderm: GATA6) in differentiated iPSC lines	Fig. 1 panel I
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	NA	NA
Genotype additional info (OPTIONAL)	Blood group genotyping	NA	NA
	HLA tissue typing	NA	NA

Scientific), N2 Supplement (Thermo Fisher Scientific). Then iPSCs colonies were transfer in MEF-free plates coated with Geltrex and fed with Essential 8 medium (Thermo Fisher Scientific). Cells were cultured at 37 °C in humidified atmosphere containing 5% CO2, feeding daily until the cells reached 80–90% confluency. Cells were passaged onto fresh Geltrex-coated plates using ReLeSR (Stem Cell Technologies) into Essential 8 medium containing 5 µM Rock kinase inhibitor Y-27632 (Tocris).

4.3. DNA sequencing

Genomic DNA was extracted from iPSCs using FlexiGene DNA Kit (Qiagen). Amplified DNA fragments were sequenced using a BigDye Terminator v3.1 sequencing kit and a 3130 Genetic Analyzer (Applied Biosystems). Primer sequences are listed in Table 3.

4.4. Comparative genomic hybridization array analysis

CGH array was performed according to previously established protocol (Khudiakov et al., 2017). For analysis FAMRCi007-A and FAMRCi007-B iPSC lines at passage 10 were used. In spite of CGH array high resolution, this technique has some limitations, particularly inability to detect chromosomal abnormalities not associated with changes in DNA copy number such as translocations or inversions, alterations in chromosome structure, mosaicism or polyploidy.

4.5. Short tandem repeats analysis

STR analysis was performed on the FAMRCi007-A and FAMRCi007-B iPSC lines using the COrDIS mini2 STR Amplification Kit (Gordiz) according to the manufacturer's recommendations. A complete match of 11 STR loci in iPSCs and patient's PBMCs is presented in the Supplementary file.

4.6. Immunofluorescence and flow cytometry

For immunofluorescent staining cells were fixed with 4% paraformaldehyde for 12 min at room temperature. Then cells were washed with PBS and permeabilized with 0.5% TritonX-100 in PBS for 5 min. For blocking of nonspecific binding cells were incubated with 1% BSA solution in PBS for 30 min and then incubated with the primary antibodies for 1 h. Then cells were washed with PBS and incubated with secondary antibody for 1 h. DAPI was used for nuclear counterstaining. Images were captured using AxioObserver Z1 (Zeiss) microscope and processed using Zen Blue software (Zeiss). In addition, expression of pluripotent surface marker SEEA4 was confirmed by flow cytometry on CytoFLEX equipment (Beckman Coulter) using CytExpert software (Beckman Coulter) for data analyzing. Antibodies used in this study are listed in Table 3.

4.7. Alkaline phosphatase staining

For the pluripotency confirmation iPSCs were stained using the sensitive substrate for alkaline phosphatase detection NBT/ BCIP (Roche) solution according to manufacturer's instructions.

4.8. RT-PCR and qPCR

Total RNA was extracted from iPSCs lines using Extract RNA reagent (Evrogen) and treated with DNaseI (Thermo Fisher Scientific). The reverse transcription of 1 µg of RNA was performed using MMLV RT kit (Evrogen) according to the manufacturer's instructions. QPCR was carried out using the qPCRmix-HS SYBR + LowROX (Evrogen) and gene-specific primers. The relative quantification of target genes was calculated by the $2^{-\Delta\Delta Ct}$ method with the *GADPH* used as reference gene. To confirm the elimination of exogenous reprogramming transgene factors in the iPSC lines RT-PCR analysis was applied with primers specific to Sendai vectors' sequences (*KOS*, *SeV*) or *GADPH* gene. Primer sequences are listed in Table 3.

4.9. In vitro trilineage differentiation

STEMdiff™ Trilineage Differentiation Kit (Stem Cell Technologies, Canada) with subsequent immunofluorescent staining against lineage-specific markers was used to validate the differentiation potential of the established iPSC lines. Used antibodies are listed in Table 3.

4.10. Mycoplasma detection

The cell culture medium debris was used as mycoplasma analysis template for PCR with gene-specific primers (Uphoff and Drexler, 2002). We observed negative results for FAMRCi007-A and FAMRCi007-B iPSC lines (Supplementary file).

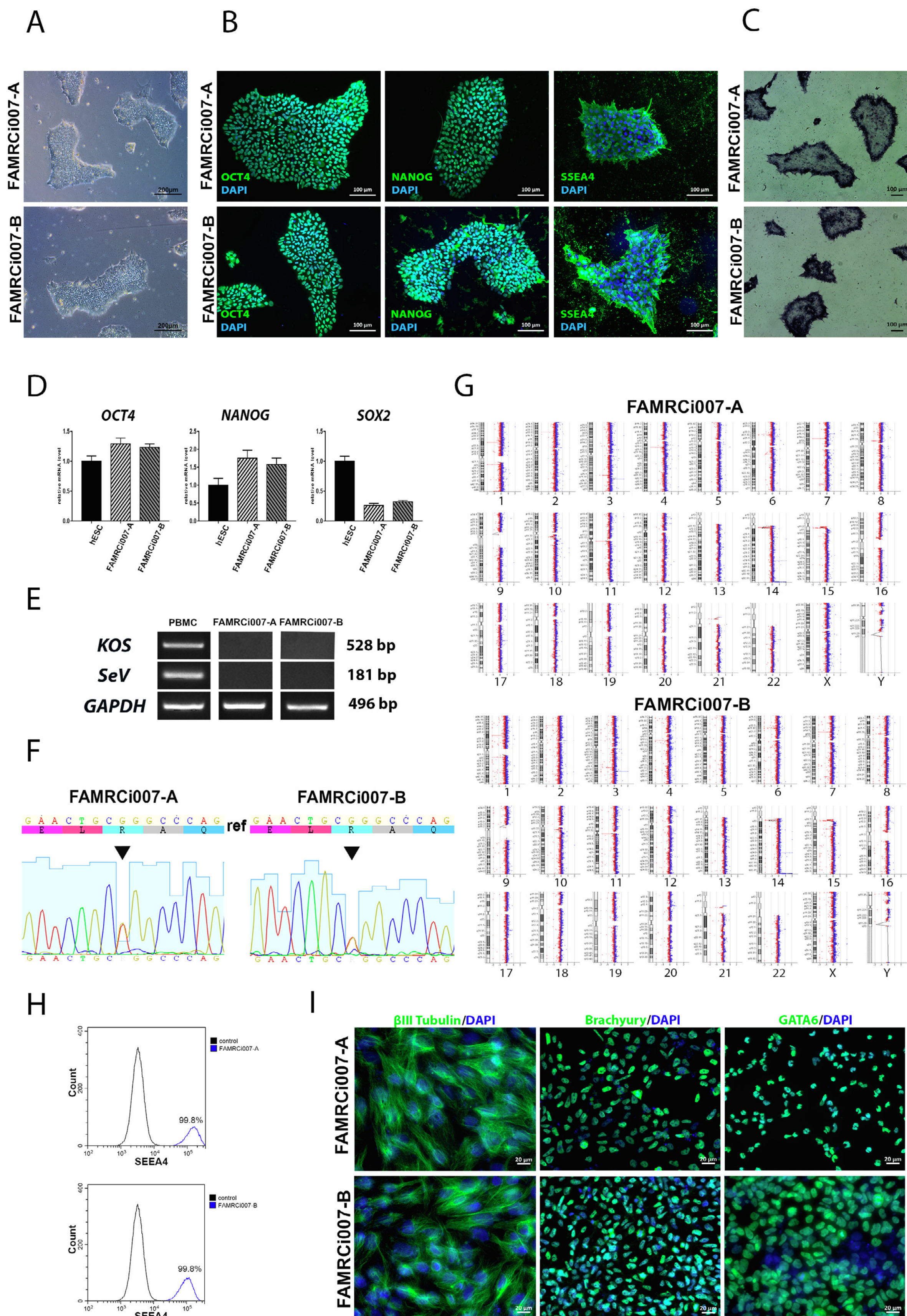

Fig. 1. Characterization of FAMRCi007-A and FAMRCi007-B iPSC lines.

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Goat anti-OCT4	1:200	Santa Cruz Cat# 9656, RRID:AB_653551
Pluripotency Markers	Mouse anti-NANOG	1:200	Millipore Cat# MABD24, RRID:AB_11203826
Pluripotency Markers	Mouse anti-SSEA4	1:200	R&D Systems Cat# MAB1435, RRID:AB_357704
Differentiation Markers	Mouse anti-βIII Tubulin	1:200	R&D Systems Cat# MAB1195, RRID:AB_357520
Differentiation Markers	Goat anti-Brachyury	1:200	R&D Systems Cat# AF2085, RRID:AB_2200235
Differentiation Markers	Goat anti-GATA6	1:200	R&D Systems Cat# AF1700 RRID:AB_2108901
Secondary antibodies	AF488 Goat Anti-Mouse IgG	1:1000	Invitrogen Cat# A11029, RRID:AB_138404
Secondary antibodies	AF488 Donkey Anti-Goat IgG	1:1000	Invitrogen Cat# A11029, RRID:AB_2534102
Primers			
	Target	Forward/Reverse primer (5′-3′)	
Sendai virus transgenes (RT-PCR)	Sendai virus *KOS* sequence	ATGCACCGCTACGACGTGAGCGC/ACCTTGACAATCCTGATGTGG	
Sendai virus genome (RT-PCR)	Sendai virus genome sequence (*SeV*)	GGATCACTAGGTGATATCGAGC/ACCAGACAAGAGTTTAAGAGATATGTATC	
Pluripotency markers (qPCR)	*OCT4*	GAAGGAGAAGCTGGAGCAAA/CTTCTGCTTCAGGAGCTTGG	
Pluripotency markers (qPCR)	*NANOG*	CAGCCCTGATTCTTCCACCAGTCCC/TGGAAGGTTCCCAGTCGGGTTCACC	
Pluripotency markers (qPCR)	*SOX2*	AACCCCAAGATGCACAACTC/GCTTAGCCTCGTCGATGAAC	
Reference gene (qPCR)	*GAPDH*	AATGAAGGGGTCATTGATGG/AAGGTGAAGGTCGGAGTCAA	
Reference gene (RT-PCR)	*GAPDH*	CAAGGTCATCCATGACAACTTTG/GTCCACCACCCTGTTGCTGTAG	
Mutation sequencing (with M13 adapter)	*LMNA* exon 4	GGCTGGTAGTGGCTCATGGA/CCTGCCACCATCTGCCTG	

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2020.101895.

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