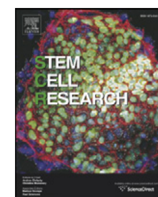
Stem Cell Research 23 (2017) 127–131

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr



Lab Resource: Stem Cell Line

Generation of a KLF15 homozygous knockout human embryonic stem cell line using paired CRISPR/Cas9n, and human cardiomyocytes derivation

Claudia Noack [a,c], Luis Peter Haupt [b,c], Wolfram-Hubertus Zimmermann [a,c], Katrin Streckfuss-Bömeke [b,c], Laura Cecilia Zelarayán [a,c,*]

[a] *Institute of Pharmacology and Toxicology, University Medical Center Goettingen, Germany*
[b] *Clinic for Cardiology and Pneumology, University Medical Center Goettingen, Germany*
[c] *DZHK (German Center for Cardiovascular Disease), Partner Site Goettingen, Germany*

ARTICLE INFO

Article history:
Received 25 May 2017
Received in revised form 25 June 2017
Accepted 7 July 2017
Available online 11 July 2017

ABSTRACT

Krueppel-like factor 15 (KLF15) is abundantly expressed in liver, kidney, and muscle, including myocardium. In the adult heart KLF15 is important to maintain homeostasis and to repress hypertrophic remodeling. We generated a homozygous hESC KLF15 knockout (KO) line using paired CRISPR/Cas9n. KLF15-KO cells maintained full pluripotency and differentiation potential as well as genomic integrity. We demonstrated that KLF15-KO cells can be differentiated into morphologically normal cardiomyocytes turning them into a valuable tool for studying human KLF15-mediated mechanisms resulting in human cardiac dysfunction.

© 2017 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Resource Table

Unique stem cell line identifier	ESIBIe002-A-1
Alternative name of stem cell line	HES2-KLF15-KO
Institution	Institute of Pharmacology and Toxicology, University Medical Center Goettingen, Germany
Contact information of distributor	Laura C. Zelarayán, laura.zelarayan@med.uni-goettingen.de
Type of cell line	ESC
Origin	Human ESC line HES2, Embryonic Stem Cell International, Singapore (ESIBIe002-A)
Additional origin info	Age: N/A Sex: female Ethnicity if known: N/A
Cell source	ESC
Method of reprogramming	Not applicable
Associated disease	Unaffected
Gene/locus	KLF15, 3q21.3
Method of modification	CRISPR/Cas9n
Gene correction	No
Name of transgene or resistance	Not applicable
Inducible/constitutive system	Not applicable
Date archived/stock date	Oct 2016
Cell line repository/bank	Not applicable
Ethical approval	German Stem Cell Act by the Robert-Koch-Institute to Wolfram-H. Zimmermann, permit #12, reference number: 1710-79-1-4-16

Resource utility

KLF15 is required for normal heart homeostasis and becomes downregulated upon cardiac hypertrophy in mice and human patients (Fisch et al., 2007; Noack et al., 2012). The HES-KLF15-KO line was generated to study the pathological mechanisms triggered by KLF15 deletion in human cardiomyocyte 2D and 3D *in vitro* models.

Resource details

The transcription factor KLF15 regulates diverse cellular processes and is mainly expressed in liver, kidney, skeletal and cardiac muscle. Particularly in the adult heart it is essential to maintain normal cardiac function (Fisch et al., 2007; Noack et al., 2012) and regulates cell proliferation, hypertrophy, and lipid metabolism (Haldar et al., 2012). Importantly, KLF15 is downregulated in human and rodent cardiomyopathies, suggesting an evolutionary conserved mechanism. To explore its role in human cardiomyocytes, we deleted KLF15 function by generating a

* Corresponding author.
E-mail address: laura.zelarayan@med.uni-goettingen.de (L.C. Zelarayán).

http://dx.doi.org/10.1016/j.scr.2017.07.007
1873-5061/© 2017 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

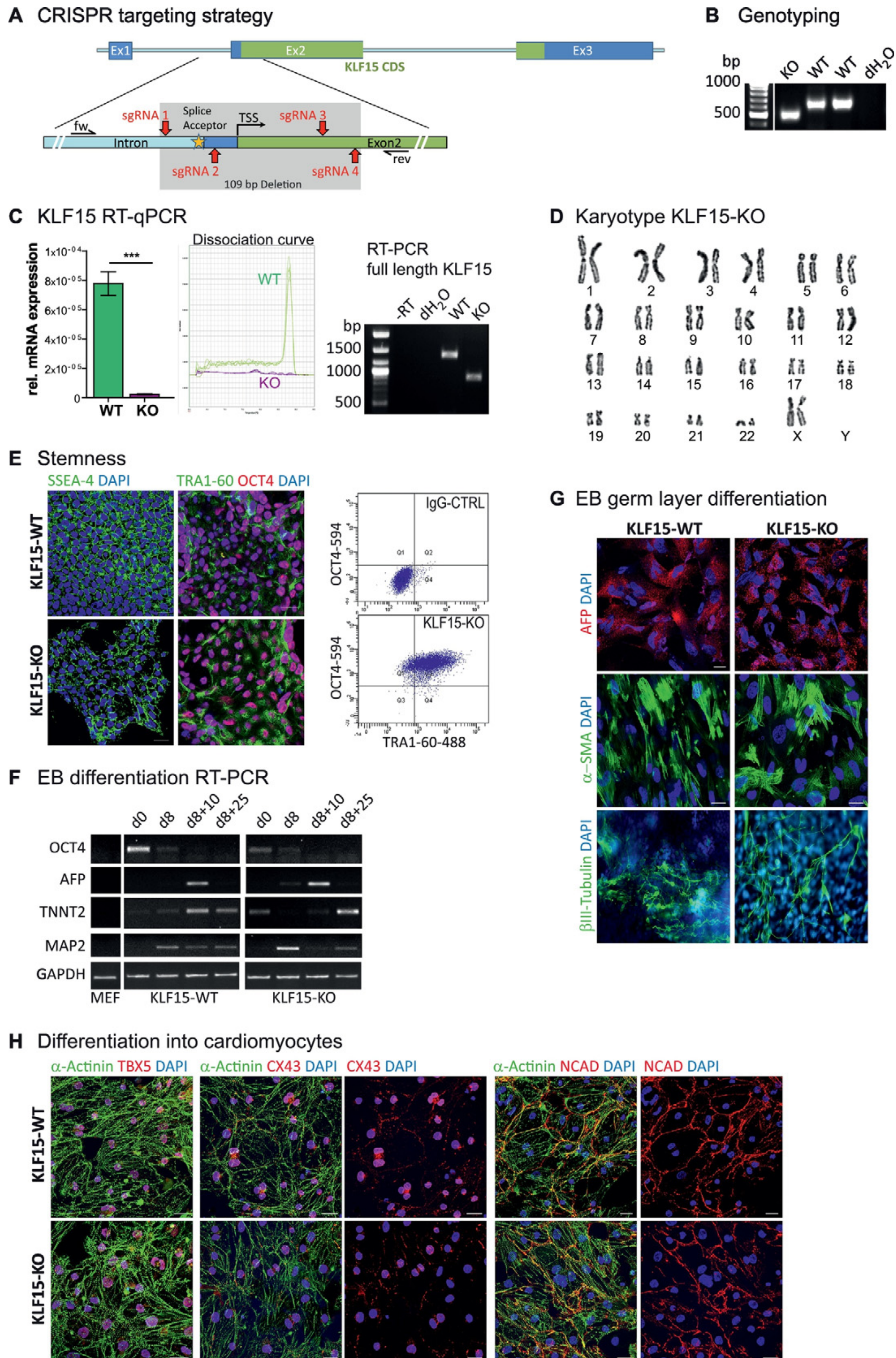


Fig. 1. Generation of the human homozygous embryonic stem cell line KLF15-knockout (KO). (A) Illustration of CRISPR/Cas9 targeting strategy for KLF15 locus. SgRNAs are indicated in red. (B) Genotyping PCR confirming the KO allele using primers (fw and rev) indicated in A. (C) RT-PCR showing the truncated transcript in the KLF15-KO line. (D) G-banding karyotype analysis of the KLF15-KO line. (E) Pluripotency assessment of KLF15-KO and -WT lines by immunofluorescence of OCT4, TRA1-60, SSEA-4 and flow cytometry of OCT4 and TRA1-60. (F and G) RT-PCR and immunofluorescence analysis of embryoid bodies (EBs) showing endodermal (AFP); mesodermal (*TNNT2*, α-SMA,); and ectodermal (*MAP2*, βIII-Tubulin) differentiation in KLF15-KO and -WT lines. (H) Immunofluorescence of α-Sarcomeric-Actinin, gap junction protein Connexin-43 (CX43), cell adhesion component N-Cadherin (NCAD), and transcription factor TBX5 in 2D cardiomyocytes differentiation in KLF15-KO line. Scale bar 20 mm.

homozygous hESC KLF15-KO line. We used a double-paired CRISPR/Cas9-D10A nickase (Cas9n) gene editing approach to minimize potential off-target effects. To target the genomic locus of *KLF15*, we designed four sgRNAs with a cutting offset of 128 bp including the splice acceptor and the transcription start site (TSS) (Fig. 1A). HES2 cells were nucleofected with all four sgRNAs expressing plasmids, also containing Cas9n and puromycin resistance. Cells were selected with puromycin and seeded at very low density to allow colony growth from single cells. After PCR genotyping (Fig. 1B, primer sites shown in Fig. 1A) and sequencing, positive colonies (KLF15-KO) were expanded and cryopreserved. Nucleofected cells having wild-type *KLF15* (KLF15-WT) locus served as controls. The lines were authenticated by STR analysis and tested negative for mycoplasma.

Due to the lack of reliable antibodies, the homozygous knockout was verified by reverse transcription and qPCR analyses of different *KLF15* locus regions. This revealed the anticipated absence of transcripts encoding for KLF15 ($n = 8$ different passages). *GAPDH* was equally expressed and used for normalization. RT-PCR confirmed the truncation of the *KLF15* transcript (Fig. 1C).

Genomic integrity was demonstrated by standard G-banding karyotype analysis of the KO line (Fig. 1D). Pluripotency was assessed by immunofluorescence using the stemness markers OCT4, TRA1-60, SSEA-4, and by flow cytometry showing 99.3% OCT4 and 88.1% TRA1-60 positive cells (Fig. 1E). Spontaneous differentiation capacity into all three germ layers was tested by formation of embryoid bodies (EBs) in the KLF15-KO and -WT line. This was demonstrated by marker expression on mRNA- and protein level of α-Feto-Protein (AFP, endodermal); cardiac TroponinT and α-Smooth Muscle Actin (*TNNT2*, α-SMA, mesodermal); and Microtubule-Associated Protein 2 (*MAP2*) as well as βIII-Tubulin (ectodermal) at different stages (Fig. 1F and G).

Finally, we investigated the suitability of the KLF15-KO line for studies in cardiomyocytes. We performed directed 2D differentiation followed by lactate selection and obtained spontaneously beating cardiomyocytes (videos provided as supplemental data). The KLF15-KO and -WT cells showed comparable expression of the cardiac markers α-Sarcomeric-Actinin, gap junction protein Connexin-43 (CX43), cell adhesion component N-Cadherin (NCAD), and transcription factor TBX5 (Fig. 1H).

In summary, we generated a homozygous KLF15-KO hESC line with normal karyotype and full pluripotency. KLF15-KO cells have the capacity to differentiate into cardiomyocytes, indicating that KLF15 is not essential for their development as shown previously in mice. Moreover, we showed that the KLF15-KO line has the potential to generate all three germ layers. Since KLF15 is important for the homeostasis of several organs, the KLF15-KO line represents a valuable tool for *in vitro* translational studies and disease modelling.

1. Materials and methods

1.1. Cell culture

The human embryonic stem cell line HES2 (Embryonic Stem Cell International, Singapore) was cultured in TeSR-E8 (StemCell Technologies) in Matrigel pre-coated flasks under standard conditions (37 °C, 5% CO_2). Cells were passaged using 0.5 mmol/L EDTA and TeSR-E8 with 10 μmol/L ROCK inhibitor Y27632 (Stemgent).

1.2. Mycoplasma detection

Regular testing for mycoplasma was performed with the MycoAlert-PLUS Mycoplasma detection Kit (Lonza) according to manufacturer's instruction. Ratios < 1 were considered as mycoplasma-free.

1.3. Gene targeting and clonal selection

CRISPR sgRNAs targeting the transcription start site (TSS) of human *KLF15* were designed and cloned into modified pX335 containing GFP-T2A-puromycin cassette and SpCas9-D10A nickase (Zhang et al., 2014).

2×10^6 HES2 were electroporated with 1 μg per sgRNA/Cas9n plasmid using the Human Stem Cell Nucleofector Kit 1 and the Nucleofector 2b device, program B16 (Lonza). After 24 h, cells were selected with 0.4 μg/mL puromycin for 1 day. After colony formation, halves colonies were manually picked and subjected to genotyping; the rest were maintained in culture. Positive colonies were expanded and cryopreserved (Table 1).

1.4. Genotyping and sequence analysis

Genomic DNA was isolated using the NucleoSpin Tissue kit. PCR genotyping was performed using Phusion polymerase (ThermoFisher) and 5% DMSO. PCR products were analyzed on agarose gels, extracted with NucleoSpin Gel and PCR Clean-Up, and sequenced at Seqlab Goettingen. Primer sequences are listed in Table 2.

Table 1
Detailed instructions for identification of the human homozygous embryonic stem cell line KLF15-KO line.

Classification	Test	Result	Data
Morphology	Photography	Normal	Not shown, but available with author
Phenotype	Immunocytochemistry	Assess staining of pluripotency markers OCT4, SSEA-4, TRA1-60	Fig. 1 panel E
	Flow cytometry	Assess antigen levels & cell surface markers OCT4: 99% TRA1-60: 88%	Fig. 1 panel E
Genotype	Karyotype (G-banding)	46, XX Resolution 400	Fig. 1 panel D
Identity	Microsatellite PCR (mPCR)	Not performed	
	STR analysis	21 loci were tested with PowerPlex 21 PCR Kit; matched	Submitted in archive with journal
Mutation analysis (if applicable)	Sequencing	Homozygous, deletion	Supplementary Fig. 1
	Southern Blot OR WGS	Not performed	
Microbiology and virology	Mycoplasma	Tested by bioluminescence Result: negative	Not shown, but available with author
Differentiation potential	Embryoid body formation OR Teratoma formation OR Scorecard	Embryoid body formation, expression of AFP, α-SMA, cTNT, βIII-Tubulin, and Microtubule Associated Protein 2 (MAP2)	Fig. 1 panel F and G
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	

Table 2
Detailed information for antibodies and primers.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Mouse anti-OCT4-Alexa-Fluor®647	1:100	BD Biosciences, Cat# 560329, RRID: AB_1645318
	Mouse anti-TRA1-60-Alexa-Fluor®488	1:100	BD Biosciences, Cat# 560173, RRID: AB_1645379
	Mouse anti-SSEA-4	1:200	Abcam, Cat# ab16287, RRID: AB_778073
Differentiation Markers	Mouse anti- α-SMA	1:500	Sigma-Aldrich, Cat# A2547, RRID: AB_476701
	Rabbit anti-AFP	1:100	Dako, Cat# A0008, RRID: AB_2650473
	Mouse anti-βIII-Tubulin	1:500	BAbCo, Cat# MMS-435P, RRID: AB_2313773
	Mouse anti-α-Actinin (sarcomeric)	1:500	Sigma-Aldrich, Cat# A7811, RRID: AB_476766
	Rabbit anti-Connexin-43	1:200	Sigma-Aldrich, Cat# C6219, RRID: AB_476857
	Rabbit anti-N-Cadherin	1:100	Santa Cruz, Cat# sc-7939, RRID: AB_647794
	Rabbit anti-TBX5	1:20	Sigma-Aldrich, Cat# HPA008786, RRID: AB_10601720
Secondary antibodies	Goat anti-mouse IgG-Alexa Fluor®488	1:250	Invitrogen, Cat# A11001, RRID: AB_2534069
	Goat anti-rabbit IgG-Alexa Fluor®594	1:250	Invitrogen, Cat# R37117, RRID: AB_2556545

Primers		
	Target	Forward/reverse primer (5′–3′)
Pluripotency marker	*OCT4*-fw	GACAACAATGAAAATCTTCAGGAGA
	OCT4-rev	TTCTGGCGCCGGTTACAGAACCA
Differentiation markers	*AFP*-fw	ACTCCAGTAAACCCTGGTGTTG
	AFP-rev	GAAATCTGCAATGACAGCCTCA
	TNNT2-fw	GACAGAGCGGAAAAGTGGGA
	TNNT2-rev	TGAAGGAGGCCAGGCTCTAT
	MAP2-fw	CCACCTGAGATTAAGGATCA
	MAP2-rev	GGCTTACTTTGCTTCTCTGA
House-keeping gene	*GAPDH*-fw2	AGAGGCAGGGATGATGTTCT
	GAPDH-rev2	TCTGCTGATGCCCCCATGTT
qPCR KO validation	*KLF15*-SA-fw	TGCGCCAAGTTCAGCCGC
	KLF15-SA-rev	GCGTGGCCTGGGACAATAGG
	KLF15-FL-fw	GGCGTGCGCCAAGTTC
	KLF15-FL-rev	TTCAGGGCGCTTTCAGTTCA
	GAPDH-fw	AAGGCTGTGGGCAAGGTCATC
	GAPDH-rev	GCGTCAAAGGTGGAGGAGTGG
DNA genotyping & sequencing	*KLF15*-fw	CAGAGAGCCTCCTCGCGCA
	KLF15-rev	GGCCTGGGACAATAGGAAGTC

1.5. RNA isolation and qPCR analysis

Total RNA was isolated using the NucleoSpin RNA kit including DNase treatment. cDNA were synthesized with Oligo $(dT)_{20}$ primer and M-MLV reverse transcriptase (Promega). qPCR analyses were performed with 1/10 of cDNA and GoTaq qPCR SYBR Green Master Mix (Promega) on a 7900-HT Real-time cycler (Applied Biosystems). Copy numbers were calculated with the relative standard curve method using the SDS2.4 software. All reactions were run in triplicates and normalized to *GAPDH*. Primer sequences are listed in Table 2.

1.6. Karyotyping

Standard G-banding (0.02% Giemsa stain, Sigma-Aldrich) of >10 metaphase spreads/line were analyzed using Case Data Manager 6.0 software on an Axio Imager M2 microscope (Zeiss).

1.7. Embryoid body (EB) differentiation

Mouse embryonic fibroblast (MEF)-plated stem cells were dispersed into small clumps with collagenase IV and cultured in suspension for 1 day. For spontaneous differentiation, the formed EBs were cultured in suspension for 1 week in differentiation medium (IMDM, 20% FCS, 2 mmol/L L-glutamine, 1× NEAA, 450 μmol/L thioglycerol). EBs at day 8 were plated on 0.1% gelatin-coated dishes and cultured up to 25 days in differentiation medium.

1.8. Immunocytochemistry

Cells and EBs at different stages were fixed with 4% PFA, permeabilized, blocked and incubated with the indicated antibodies (listed in Table 2). Nuclei were counterstained with 0.2 ng/mL DAPI. Coverslips were mounted with VectaShield mounting medium (VECTOR Laboratories). Images were acquired with a LSM 710 NLO confocal microscope (Zeiss).

1.9. Flow cytometry analysis

Cells were fixed with 4% PFA, washed in PBS, strained (70 μm), blocked and permeabilized. Fluorophore-conjugated primary antibodies (listed in Table 2) were incubated in blocking buffer with 5 μg/mL Hoechst 33342; cells were washed and resuspended in PBS. Analysis was performed with a LSRII flow cytometer and FACSDiva software, version 6.1.3 (BD).

1.10. Differentiation into cardiomyocytes

Cardiomyocytes were differentiated according to the protocol from (Tiburcy et al., 2017). Briefly, stem cells were cultured in basal medium (RPMI, 2% B27, 200 μmol/L L-ascorbic acid, 1 mmol/L Na-pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin). For mesodermal induction 9 ng/mL Activin A (R&D systems), 1 μmol/L CHIR99021, 5 ng/mL BMP4, and 5 ng/mL β-FGF (all Stemgent) were added for 3 days. For cardiac differentiation 5 μmol/L IWP4 (Stemgent) was added for days 4–13, followed by metabolic selection medium (RPMI without glucose and glutamine, 2.2 mmol/L Na-lactate, 100 μmol/L β-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin) for 5 days.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2017.07.007.

Acknowledgements

We thank Daria Reher, Iris Quentin, and Sandra Georgi for superb technical support. We thank Dr. Boris Greber for generously providing material and protocols. This work was supported by the DFG grant ZE300-3 to LCZ; SFB1002 C04 and S1 to WHZ and C07 to LCZ; and the DZHK (German Center for Cardiovascular Disease).

References

Fisch, S., Gray, S., Heymans, S., Haldar, S.M., Wang, B., Pfister, O., Cui, L., Kumar, A., Lin, Z., Sen-Banerjee, S., Das, H., Petersen, C.A., Mende, U., Burleigh, B.A., Zhu, Y., Pinto, Y.M., Liao, R., Jain, M.K., 2007. Kruppel-like factor 15 is a regulator of cardiomyocyte hypertrophy. Proc. Natl. Acad. Sci. U. S. A. 104, 7074–7079.

Haldar, S.M., Jeyaraj, D., Anand, P., Zhu, H., Lu, Y., Prosdocimo, D.A., Eapen, B., Kawanami, D., Okutsu, M., Brotto, L., Fujioka, H., Kerner, J., Rosca, M.G., McGuinness, O.P., Snow, R.J., Russell, A.P., Gerber, A.N., Bai, X., Yan, Z., Nosek, T.M., Brotto, M., Hoppel, C.L., Jain, M.K., 2012. Kruppel-like factor 15 regulates skeletal muscle lipid flux and exercise adaptation. Proc. Natl. Acad. Sci. U. S. A. 109, 6739–6744.

Noack, C., Zafiriou, M.P., Schaeffer, H.J., Renger, A., Pavlova, E., Dietz, R., Zimmermann, W.H., Bergmann, M.W., Zelarayan, L.C., 2012. Krueppel-like factor 15 regulates Wnt/beta-catenin transcription and controls cardiac progenitor cell fate in the postnatal heart. EMBO Mol. Med. 4, 992–1007.

Tiburcy, M., Hudson, J.E., Balfanz, P., Schlick, S., Meyer, T., Chang Liao, M.L., Levent, E., Raad, F., Zeidler, S., Wingender, E., Riegler, J., Wang, M., Gold, J.D., Kehat, I., Wettwer, E., Ravens, U., Dierickx, P., van Laake, L.W., Goumans, M.J., Khadjeh, S., Toischer, K., Hasenfuss, G., Couture, L.A., Unger, A., Linke, W.A., Araki, T., Neel, B., Keller, G., Gepstein, L., Wu, J.C., Zimmermann, W.H., 2017. Defined engineered human myocardium with advanced maturation for applications in heart failure modeling and repair. Circulation 135, 1832–1847.

Zhang, M., D'Aniello, C., Verkerk, A.O., Wrobel, E., Frank, S., Ward-van Oostwaard, D., Piccini, I., Freund, C., Rao, J., Seebohm, G., Atsma, D.E., Schulze-Bahr, E., Mummery, C.L., Greber, B., Bellin, M., 2014. Recessive cardiac phenotypes in induced pluripotent stem cell models of Jervell and Lange-Nielsen syndrome: disease mechanisms and pharmacological rescue. Proc. Natl. Acad. Sci. U. S. A. 111, E5383–E5392.