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Lab Resource: Genetically-Modified Multiple Cell Lines

Generation of bi-allelic *MYBPC3* truncating mutant and isogenic control from an iPSC line of a patient with hypertrophic cardiomyopathy

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A B S T R A C T

MYBPC3 is the most frequently affected gene in hypertrophic cardiomyopathy (HCM), which is an autosomal-dominant cardiac disease caused by mutations in sarcomeric proteins. Bi-allelic truncating *MYBPC3* mutations are associated with severe forms of neonatal cardiomyopathy. We reprogrammed skin fibroblasts from a HCM patient carrying a heterozygous *MYBPC3* truncating mutation into human induced pluripotent stem cells (iPSC) and used CRISPR/Cas9 to generate bi-allelic *MYBPC3* truncating mutation and isogenic control hiPSC lines. All lines expressed pluripotency markers, had normal karyotype and differentiated into endoderm, ectoderm and cardiomyocytes in vitro. This set of three lines provides a useful tool to study HCM pathomechanisms.

Resource Table

Unique stem cell lines identifier	1. UKEi070-A 2. UKEi070-A1 3. UKEi070-A2
Alternative name(s) of stem cell lines	1. Het 2. Iso Co 3. cpHet
Institution	Institute of Experimental Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
Contact information of the reported cell line distributor	Prof. Dr. Lucie Carrier; l.carrier@uke.de
Type of cell lines	iPSC
Origin	Human
Additional origin info	Age: 53 Female Central America
Cell Source	Dermal fibroblasts
Method of reprogramming	Non-integrating Sendai virus vectors (CytoTune ™, expression of OCT-4, SOX2, c-MYC, KLF4)
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	RT-qPCR Sendai virus – negative at Master Cell Bank stage
Cell culture system used	Conditioned medium (young clones), maintenance in FTDA culture medium
Type of Genetic Modification	Induced homozygous mutation (bi-allelic mutant hiPSC line) and gene correction (isogenic control)
Associated disease	Hypertrophic cardiomyopathy (CMH4; 115197)
Gene/locus	*MYBPC3*/chr11 (p11.2): - Heterozygous c.2308G>A, p. Asp770Serfs98X (patient)

(*continued on next column*)

Resource Table (*continued*)

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Resource Table (*continued*)

	- Isogenic control for c.2308G+6 G>T (wild-type plus silent mutation) - Compound heterozygous: c.2308G>A, p.Asp770Serfs98X (heterozygous) and c.2827C>T, p.Arg943Ter (homozygous)
Method of modification/site-specific nuclease used	CRISPR/Cas9
Site-specific nuclease (SSN) delivery method	Nucleofection of RNP
All genetic material introduced into the cells	RNP complex (crRNA, tracrRNA and Cas9 nuclease) and 120-mer ssODNs for HDR
Analysis of the nuclease-targeted allele status	Sanger sequencing of the targeted locus and bacterial subcloning
Method of the off-target nuclease activity surveillance	Targeted PCR and Sanger sequencing of the Top10 off-target loci
Name of transgene	N/A
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	N/A
Inducible/constitutive system details	N/A
Date archived/stock date	UKEi070-A (Het): 11/04/2018 UKEi070-A-1 (Iso Co): 19/07/2018 UKEi070-A-2 (cpHet): 14/09/2018
Cell line repository/bank	No repository/bank
Ethical/GMO work approvals	The patient gave her written informed consent for research purposes and the study was approved by the ethical committee of the UKE, Hamburg (PV3501).
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	N/A

1. Resource utility

The three hiPSC lines provide an everlasting source for differentiation into cardiomyocytes for HCM disease modelling. The combination of heterozygous and compound heterozygous *MYBPC3* truncating mutations with their isogenic control allows to study the effect of *MYBPC3* gene dosage on HCM pathomechanisms and to test new therapeutic options.

2. Resource details

HCM is widely heterogenous in its clinical manifestations, ranging from no symptoms to sudden death. Most patients carry heterozygous mutations and have a late onset of the disease. In contrast, a minority of patients carrying compound heterozygous or homozygous truncating *MYBPC3* mutations leading to the complete absence of protein presents with a severe cardiomyopathy already at birth, which rapidly develops into heart failure and death within the first year of life (reviewed in Carrier, 2021). Heart transplantation remains the only curative treatment for these infants. The pathogenic heterozygous *MYBPC3* mutation (c.2308G>A; p.Asp770Serfs98X) was identified in a HCM patient recruited in the outpatient clinic at the University Heart Center Hamburg (Table 1). This mutation is located on the last nucleotide of exon 23 and is considered truncating (Helms et al., 2014). Patient-derived fibroblasts were reprogrammed into hiPSC with Sendai Virus and used as parental line (Het) to generate *MYBPC3*-corrected iPSC line (isogenic control, Iso Co) via CRISPR/Cas9 genome editing. In addition, the Dutch founder nonsense mutation c.2827C>T, p.Arg943Ter (Christiaans et al., 2010) was introduced at the homozygous state in Het hiPSC to generate a bi-allelic mutant (cpHet) hiPSC line, to recapitulate the genetic situation of infants with severe forms of cardiomyopathy (Fig. 1A).

The three hiPSC lines were validated for essential features (Table 2). They showed a typical stem cell morphology and normal karyotypes (Fig. 1B, C). Sanger sequencing confirmed the heterozygous mutation in Het, and the expected CRISPR/Cas9 genetic modifications of the Iso Co and cpHet hiPSC lines (Fig. 1D). FACS analysis revealed a high percentage of positive cells for the pluripotency marker SSEA3 in all lines (>80%; Fig. 1E), and RT-PCR analysis showed the expression of other pluripotency markers, *NANOG* and *SOX2* (Fig. 1F). Extensive evaluation of differentiation potential has been performed for the parental Het line comparing by RT-qPCR gene expression of specific markers before and after differentiation (Fig. 1F). In addition specific expression in the relative lineages has been verified by RT-PCR in Het and in the two subclones Iso Co and cpHet (Fig. 1G). Furthermore the hiPSC lines were capable of differentiating into cardiomyocytes as shown by the high percentage of cardiac troponin T (cTnT)-positive cells (Fig. 1H). Off-target analysis showed no effects in the Top10 analysed loci, suggesting high fidelity of the RNP-based CRISPR approach (Suppl. Fig. 1). STR analysis proved that the genetically modified hiPSC lines originate from the Het (data provided to hiPSCreg). Protein lysate of hiPSC-derived cardiomyocytes (hiPSC-CMs) showed complete absence of MYBPC3 protein in cpHet (Fig. 1I).

3. Materials and methods

3.1. Reprogramming and culture

Dermal fibroblasts were obtained from a skin biopsy taken from the HCM patient and were used for reprogramming with the CytoTune-iPS Sendai Reprogramming Kit (#A1377801, Life Technologies) according to the manufacturer's instructions. The hiPSCs were cultured on Matrigel-coated plates containing conditioned medium. Passaging was performed twice a week with Accutase™ supplemented with Rock inhibitor (10 μM Y-27632). The medium was replaced daily and hiPSC were cultured under hypoxic conditions (37 °C, 5% CO_2 and 5% O_2). From passage 19 onwards, hiPSC were adapted to FTDA culture medium and cultured on Geltrex-coated culture plates. Master cell banks were created as previously described (Shibamiya et al. 2020).

3.2. Genetic modification using CRISPR/Cas9

For genetic modification two gRNAs were designed with the CRISPR design tool http://crispr.mit.edu/ (Table 3). Electroporation was performed in hiPSC Het (passage 19) using the 4D-Nucleofector™ System (Lonza). Nucleofected cells were plated on Matrigel-precoated 12-well plates and after 48 h gently detached in the presence of 10 μM Y-27632, seeded at low density and further cultured in conditioned-medium. Single cell-derived colonies were picked, replated and

Table 1
Summary of lines.

iPSC line Name	Abbreviation in figure	Gender	Age	Ethnicity	Genotype of locus	Disease
UKEi070-A	Het	Female	53	Central America	c.2308 G>A heterozygous	HCM
UKEi070-A1	Iso Co	Female	53	Central America	c.2308 G homozygous c.2308 G>T (silent) heterozygous	
UKEi070-A2	cpHet	Female	53	Central America	c.2308 G>A heterozygous c.2827C>T homozygous	

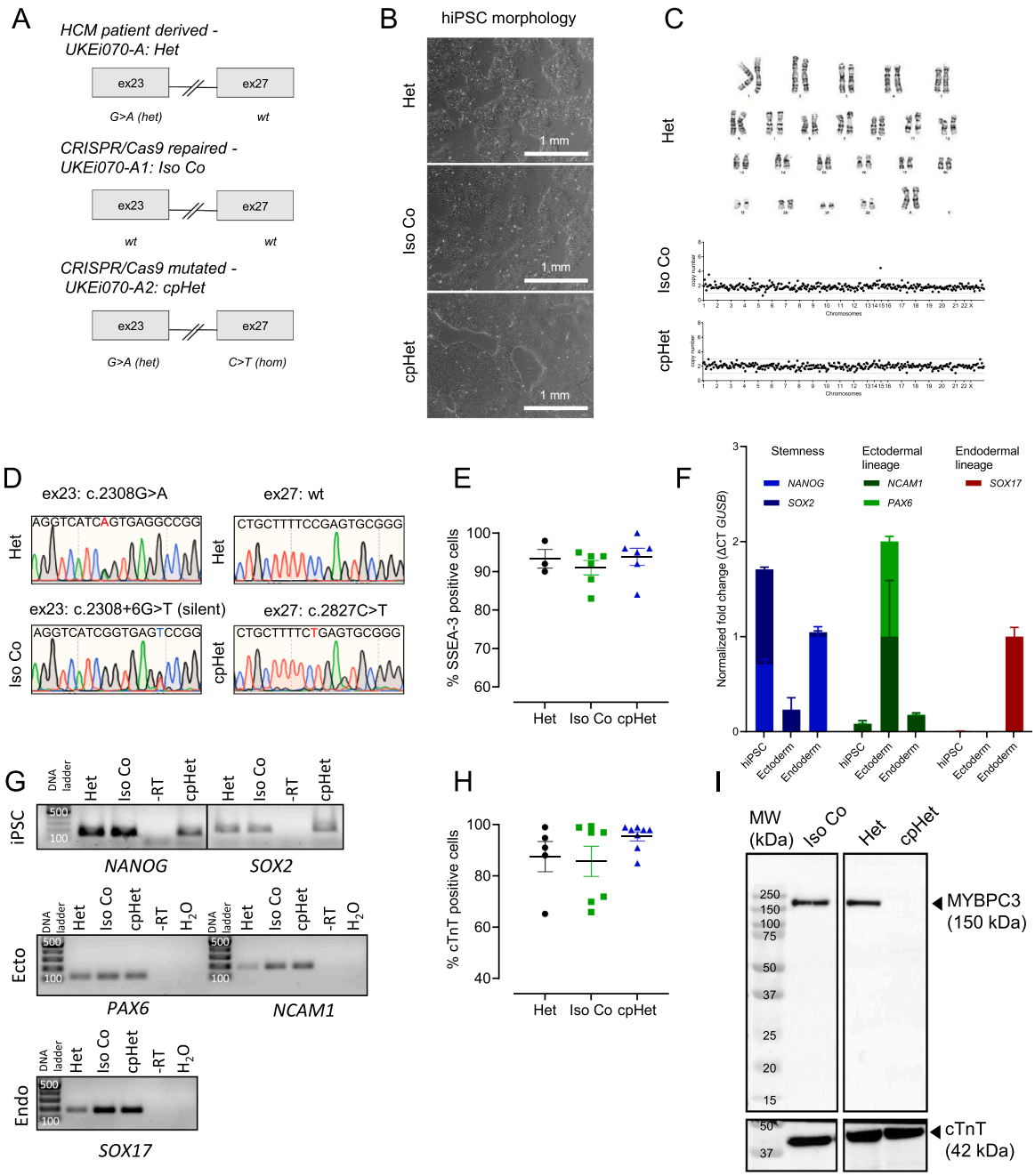

Fig. 1. Characterization of hiPSC lines UKEi070-A (Het), UKEi070-A1 (Iso Co) and UKEi070-A2 (cpHet). A) Schematic depiction of *MYBPC3* genetic status in the three lines. B) Normal morphology of the hiPSC colonies. C) G-Banding for the patient-specific cell line (Het) and NanoString nCounter human karyotype analysis after CRISPR/Cas9 genome editing revealed regular female karyotypes. D) Validation of the genetic status by Sanger sequencing of the three hiPSC lines. E) Flow cytometry analysis of the pluripotency marker SSEA-3 in several batches of hiPSC lines. F) RT-qPCR of the patient-specific cell line (Het) confirming specific gene expression on mRNA level. G) RT-PCR of pluripotency markers (*NANOG* and *SOX2)* in all three iPSC lines and markers of ectoderm (*PAX6* and *NCAM1*) and endoderm (*SOX17*) after respective differentiation in vitro. H) Flow cytometry analysis of cardiac troponin T (cTnT)-positive cells after cardiac differentiation. I) Western blot of protein lysate from cardiomyocytes shows the absence of MYBPC3 in cpHet, confirming the bi-allelic truncating mutation.

eventually cryopreserved. For genotyping DNA was extracted using the QIAcube® HT workstation (Qiagen) and the QIAamp® 96 DNA QIAcube® HT Kit (Qiagen) according to the manufacturer's instruction. PCR amplification was performed with the DreamTaq DNA Polymerase (Thermo Fisher Scientific) and amplicons sequenced.

3.3. Karyotyping

Karyotype integrity of Het hiPSC was investigated at passage 6 by G-banding of 15 metaphases. Karyotypes of genetically modified hiPSC were verified with the NanoString nCounter® Human Karyotype Panel following the manufacturers protocol (passage 31 Iso Co, passage 33 cpHet).

3.4. Flow cytometry

For determination of pluripotency and cardiac differentiation by flow cytometry, we used anti-SSEA-3 and anti-cardiac troponin T (cTnT) antibodies, respectively, and compared to corresponding isotype controls. Analysis was performed in the UKE FACS Core Facility using the

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	A visual record of the line's cellular morphology: typical pluripotent human stem cell morphology	Fig. 1 panel B
Pluripotency status evidence for the described cell line	Qualitative and quantitative Reverse Transcriptase - PCR Quantitative analysis: Flow cytometry	Expression of pluripotent markers (*NANOG* and *SOX2),* ectoderm (*NCAM1* and *PAX6*) and endoderm (*SOX17*) Assess % of positive cells for cell surface marker SSEA-3	Fig. 1 panel G, Fig. 1 panel F Fig. 1 panel E
Karyotype	G-banding nCounter® Human Karyotype Panel	Normal karyotype: 46, XX	Fig. 1 panel C
Genotyping for the desired genomic alteration/ allelic status of the gene of interest	PCR across the edited site	PCR + Sanger sequencing: - Isogenic control: correction of c.2308G>A and introduced silent mutation - Bi-allelic mutant: homozygous c.2827C>T	Fig. 1 panel D
	Transgene-specific PCR	N/A	N/A
Verification of the absence of random plasmid integration events	PCR	N/A	N/A
Parental and modified cell line genetic identity evidence	STR analysis	Identical for: *SE33* *D21S11* *VWA* *TH01* *FGA* *D3S1358* *D8S1179* *D18S51* *D1S1656* *D2S441* *D10S1248* *D12S391* *D22S1045* *D16S539* *D2S1338* *D19S433* *Amelogenin*	Provided, not shown
Mutagenesis/genetic modification outcome analysis	Sequencing (genomic DNA PCR) PCR-based analyses Western Blot	Sanger sequencing tracks Not performed Absence of MYBPC3 in cpHet	Fig. 1 panel D Not performed Fig. 1 panel D
Off-target nuclease analysis	PCR across the top ten predicted likely off-target sites	Demonstration of the lack of NHEJ-caused mutagenesis in the top ten predicted off-target sites	Supplemental Fig. 1
Specific pathogen-free status	Mycoplasma	PCR	Not shown
Multilineage differentiation potential	Directed differentiation	Cardiac differentiation (mesoderm), Flow cytometry STEMdiff Trilineage Differentiation Kit	Fig. 1 panel H Fig. 1 panel G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	HIV 1 Taq-PCR QL, HBV-PCR HCV-PCR QL on primary fibroblasts under detection threshold	Not shown
Genotype – additional histocompatibility info (OPTIONAL)	Blood group genotyping HLA tissue typing	Not performed Not performed	Not performed Not performed

FACSCanto II Flow Cytometer.

3.5. Germ layer differentiation

To verify the differentiation capacity of the hiPSC lines into all three germ layers, the STEMdiff Trilineage Differentiation Kit (StemCell Technologies, #05230) was used according to the manufactureŕs instructions for endoderm and ectoderm. Mesodermal differentiation was shown by directed cardiac differentiation in a monolayer format.

3.6. RNA isolation and RT-(q)PCR

Total RNA was isolated from hiPSCs and differentiated cells with Trizol (Life Technologies), following the manufactureŕs protocol. Reverse transcription of 200–500 ng of RNA was performed using SuperScript III First-Strand Synthesis System (Invitrogen) and oligd(T) as described in the datasheet. RT-(q)PCR was done for each gene of interest and beta-glucoronidase (*GUSB)* was used as housekeeping gene.

3.7. Western blot

For Western blot analysis protein lysates from hiPSC-CMs were separated on 10% SDS-polyacrylamide (29:1) mini-gels and transferred to nitrocellulose membrane. Used antibodies are listed in Table 3. Detection was performed with the Clarity Western ECL Substrate (Bio-Rad) and visualised with the ChemiDoc™ Touch Imaging System (Bio-Rad).

3.8. Mycoplasma

Absence of mycoplasma contamination was routinely assessed by PCR as previously described (Shibamiya et al., 2020).

Table 3
Reagents details.

	Antibody	Dilution	Company Cat # and RRID
Pluripotency Marker	Rat Anti-Human SSEA-3 Antibody, PE Conjugated Rat IgM, k antibody, BD Biosciences	1:50	BD Biosciences Cat# 560237, RRID:AB_1645542 BD Biosciences Cat# 553943, RRID:AB_10056839
Cardiac differentiation marker	Cardiac Troponin T Antibody, anti-human/mouse/rat, REAfinity™ REA Control Antibody (I), human IgG1, REAfinity™	1:50	Miltenyi Biotec Cat# 130-119-674, RRID:AB_2751795 Cat# 130-120-709, RRID:AB_2784399
Western blot	Anti-MYBPC3 Anti-cTnT Anti-mouse IgG HRP-conjugated	1:2,000 1:3,000 1:6,000	Santa Cruz Biotechnology Cat# sc-137181, RRID:AB_2017318 Abcam Cat# ab8295, RRID:AB_306445 Sigma-Aldrich Cat# A9044, RRID:AB_258431

Site-specific nuclease		
Nuclease information	Alt-R® S.p. Cas9	Nuclease 3NLS, 2 nmol, Cat# 1081058
Delivery method	Nucleofection of the RNP complex (crRNA, tracrRNA) and Electroporation Enhancer	Alt-R® CRISPR-Cas9 crRNA, 2 nmol (IDT®); Alt-R® CRISPR-Cas9 tracrRNA, ATTO™ 550, 5 nmol: Cat# 1075927 (IDT®); Alt-R® Cas9 Electroporation Enhancer, 10 nmol: Cat# 1075916 (IDT®)
Selection/enrichment strategy	N/A	N/A
Primers and Oligonucleotides used in this study	**Target**	**Forward/Reverse primer (5′-3′)**
Reprogramming Vector (RT-qPCR)	Sendai vector	F: GGATCACTAGGTGATATCGAGC R: ACCAGACAAGAGTTTAAGAGATATGTATC
Pluripotency Markers (RT-(q)PCR)	*NANOG* (stem cell)	F: GATTTGTGGGCCTGAAGAAA R: AAGTGGGTTGTTTGCCTTTG
	SOX2 (stem cell)	F: AGTCTCCAAGCGACGAAAAA R: TTTCACGTTTGCAACTGTCC
Differentiation markers (RT-(q)PCR)	*SOX17* (endodermal)	F: CGCACGGAATTTGAACAGTA R: GGATCAGGGACCTGTCACAC
	PAX6 (ectodermal)	F: TGGGCAGGTATTACGAGACTG R: ACTCCCGCTTATACTGGGCTA
	NCAM1 (ectodermal)	F: ATGGAAACTCTATTAAAGTGA ACCTG R: TAGACCTCATACTCAGCATT CCAGT
Housekeeping gene (RT-qPCR)	GUSB	F: AAACGATTGCAGGGTTTCAC R: CTCTCGTCGGTGACTGTTCA
Mycoplasma detectione.g. Genotyping (desired allele/transgene presence detection)	PCR specific for the targeted allele	F: TGCACCATCTGTCACTCTGTTAACCTC R: ACTCCTACGGGAGGCAGCAGTA
Targeted mutation analysis/sequencing	Sequencing data from both alleles	Isogenic control: exon 23 F: GGCCTCTGGGGTCTGACT R: AGGCGGCTCCCACTGTACT Bi-allelic mutant: exon 27 F: GCTGACAGAGCACACATCGA R: TTCTGGGCAGAGCATTCTGG
crRNA sequence + *PAM*	Isogenic crRNA + ***PAM***: CACAGTCAAGGTCATCAGTG ***AGG*** Bi-allelic mutant crRNA + *PAM*: CCATATTGTGTGCCCGCACT ***CGG***	
Genomic target sequence	MYBPC3 Isogenic crRNA + ***PAM***: CACAGTCAAGGTCATCAGTG ***AGG*** Bi-allelic mutant crRNA + *PAM*: CCATATTGTGTGCCCGCACT ***CGG***	NC_000011.10 (47331406..47352702, complement
Top10 off-target (OT) mutagenesis predicted site sequencing primers	Isogenic control + ***PAM***: OT1: CAGGCTCAAGGTCATCAGTG ***AGG***	Isogenic control: OT1 F:CTGCCAAGAGGACCACAAGT

(*continued on next page*)

Table 3 (*continued*)

	OT2: CACAGTCAATGTCAACAGTG ***TGG*** OT3: TAAAGCCAAGGTCATCAGTG ***AAG*** OT4: CATTGTCAAGGACATCAGTG ***GGG*** OT5: GCCCTTCAAGGTCATCAGTG ***TGG*** OT6: CACAGTCAAGGACATCAGGG ***CAG*** OT7: CAGAGTCAAAGTCATCAGGG ***TAG*** OT8: CAGAATGCAGGTCATCAGTG ***CAG*** OT9: CACTGTAAAGGCCATCAGTG ***TGG*** OT10: CTCTCTCATGGTCATCAGTG ***TAG***	R:AGTGGTTGTTGCTCTTCCCG OT2 F:GTCAGGGTGGAGGAGACAGA R: GCAACACCGGAAACTAGGAG OT3 F: TCATGATGGGTGGAGGTTGG R:TCTGGAAGTTCCTTGAAGGGG OT4 F: AGTTGGCCATGGCTGCTTAT R: GGGGTGAACGAGTGCTTCTT OT5 F: GCATTTTGGGGTGTTAAAAGTGC R: TGAACTTGAGTGCAAGGCAGA OT6 F: TGGTTAGCTCATGTGCCACT R: TGTGTGGAACAGCTGAGCAT OT7 F: CCATCAGGACTGAGGGAAGC R: AAGGTGGGGTGGTCCTCTTA OT8 F: ACTGGAGGCCTTGGGAAAAA R: 5'-TCCGAGGGACCAGATCACTG OT9 F: GGGAGGATGGGGGAAAATAGC R: CAGCAGAAAGACTGCCTCGG OT10 F: CAGTGGCCTCTAAATCGGGG R: CTGAGAGGCGGATAGAAGCC
	Bi-allelic mutant: OT1: CCAAGTTGTGTGCCTGCACT ***CAG*** OT2: CAATATTGTATGCCTGCACT ***AAG*** OT3: GCTTATTTTGTGCCCGAACT ***TAG*** OT4: ACAAATTTTGTGCCCGCATT ***GAG*** OT5: CCACAGTGTGTGCCTGCACT ***CGG*** OT6: CCTTATTGGCAGCCCGCACT ***CGG*** OT7: CTATATTTTCTGCCAGCACT ***GGG*** OT8: CCATTTTGTTCACCCGCACT ***TGG*** OT9: CCTTAATGTCTGCCCGTACT ***TGG*** OT10: CCCTGGTGTGTGCCCGCAAT ***GGG***	Bi-allelic mutant: OT1 F: TTACTTGGCCGTGTAGCCTG R: CCATGGCTTCATGAGTAGGC OT2 F: AGGGCTAAATCCATCAGCACC R: TGGTGTATCCTGGACCATGC OT3 F: CATTTACAGGCGTACTTCGCA R: TCAGCATTTGTGAAAAGTGTCCC OT4 F: GCTTCAAAGGGTACAGAGCTA R: AGAAGGCAGGAAAAGATGAGTG OT5 F: GCTAAGGAGCCTGTGTGGTT R: TATCCCTACGGGAAGCCCAA OT6 F: GAGGAGGAGGATGGCTCTGA R: TCGGGGAAGTCGCCTACA OT7 F: TCCGAGTGAGCAAATCGCAT R: AGCAGGCCACTTTTCAGTGT OT8 F: TGGTAACTTCCACTCGCTGT R: GGCATCAGGTATCAGGTGTGT OT9 F: AAAGCAAAGCAGTGGGTCCT R: CCCCTCCCCTGCACATAGTA OT10 F: CTGCCTCCTTCCTGACTTGG R: AGACTCTACAGGGGCTGAGG
120 mer ssODNs used as templates for HDR-mediated site-directed mutagenesis in antisense direction (5' à 3')	Isogenic control template (+ silent mutation): CGGATGGGCCCTCCTTGGGGCTGC CCCTCTGTGTTCTCCAGCTTGGACCCCGGCCGGACTCACCGATGACCTTGACTGTGA GGTTTACCTGGTCCTCGCCCACAGGGTTCTTCACTGTGA Bi-allelic mutant template: ACTGTCACCGGCTCCGTGGTGGTAACCGGGGCTCCAGGCCCTGCCATATTGTGTGCC CGCACTCAGAAAAGCAGCCGGGCCCCCGTGGGCAGGTCCTTCACCAGTATTGATGTG TGCTCT	

3.9. Short tandem repeat analysis

STR analysis was performed with genomic DNA at the Center for Diagnostic, Institute of Legal Medicine UKE.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: GM, TE and LC hold a patent on gene-therapy vectors for treating cardiomyopathy that was licensed to DiNAQOR AG. TE and LC are members of the DiNAQOR Scientific Advisory Board and have shares in DiNAQOR.

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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.2021.102489.

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