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Lab Resource: Genetically-Modified Single Cell Line

Generation of a CRISPR/Cas edited human induced pluripotent stem cell line DHMi005-A-1 carrying a patient-specific disease-causing point mutation in the *TBX5* gene

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A B S T R A C T

A number of mutations in the human *TBX5* gene have been described which cause Holt-Oram syndrome, a severe congenital disease associated with abnormalities in heart and upper limb development. We have used a prime-editing approach to introduce a patient-specific disease-causing *TBX5* mutation (c.920_C > A) into an induced pluripotent stem cell (iPSC) line from a healthy donor. The resulting iPSC line provides a powerful tool to identify and analyze the biological and molecular impact of this specific *TBX5* mutation in comparison to the isogenic control iPSC line during cardiac development.

Resource Table

Unique stem cell line identifier	DHMi005-A-1 https://hpscreg.eu/cell-line/DHMi005-A-1
Alternative name(s) of stem cell line	L_mut
Institution	Department of Cardiovascular Surgery, Institute Insure, German Heart Center Munich
Contact information of the reported cell line distributor	Harald Lahm, lahm@dhm.mhn.de
Type of cell line	iPSC
Origin	human
Additional origin info *(applicable for human ESC or iPSC)*	Age: 29 years Sex: male Ethnicity: Caucasian
Cell Source	Adipose fibroblasts
Method of reprogramming	CytoTune-iPS^{TM}-iPS 2.0 Sendai Reprogramming (Invitrogen, Thermo Fisher Scientific)
Clonality	Clonal N/A

(*continued on next column*)

Resource Table (*continued*)

Evidence of the reprogramming transgene loss (including genomic copy if applicable)	
Cell culture system used	Feeder-free on Matrigel-coated plates
Type of Genetic Modification	Induced mutation in exon 4 (c.920_C > A) of the *TBX5* gene by CRISPR/Cas
Associated disease	Holt-Oram Syndrome (HOS), OMIM #142900
Gene/locus	*TBX5*, 12q24.21
Method of modification/site-specific nuclease used	Prime editor: fusion protein of Cas nickase domain (RuvC, HNH nuclease inactivated) and engineered RT domain (pentamutated)
Site-specific nuclease (SSN) delivery method	Electroporation
All genetic material introduced into the cells	pCMV-PE2 (Addgene #132775); pU6-pegRNA-GG-acceptor (Addgene #132777) with specific sgRNA, scaffold and prime-editing sequence inserted; BPK1520 (Addgene #65777)

(*continued on next page*)

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Resource Table (*continued*)

	with specific nick sgRNA sequence inserted
Analysis of the nuclease-targeted allele status	Sequencing of the targeted allele
Method of the off-target nuclease activity surveillance	Targeted PCR
Name of transgene	N/A
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	None, manual selection of single colonies
Inducible/constitutive system details	N/A
Date archived/stock date	December 2021
Cell line repository/bank	https://hpscreg.eu/cell-line/DHMi005-A-1
Ethical/GMO work approvals	Ethical committee of the Medical Faculty of the Technical University of Munich, #5943/13
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	pCMV-PE2 (Addgene #132775) (RRID: Addgene_132775); pU6-pegRNA-GG-acceptor (Addgene #132777) (RRID: Addgene_132777); BPK1520 (Addgene #65777) (RRID: Addgene_65777)

1. Introduction

1.1. Resource utility

TBX5 mutations are causative for the development of Holt-Oram syndrome, a congenital disease characterized by malformations of the anterior extremities and cardiac dysfunctions. We introduced a patient-specific *TBX5* mutation in iPSCs derived from a healthy donor to establish a disease-model to analyze the biological consequences of this particular mutation.

1.2. Resource details

In this study we have introduced a deleterious patient-specific *TBX5* point mutation (c.920_C > A) (Dreßen et al., 2016) located in exon 4 (Resource Table) in a human iPSC line (DHMi005-A, DOI:10.1016/j.scr.2022.102662) derived from a healthy proband which has been fully characterized. To perform the transversion mutation C → A we used a prime editing approach which avoids double-strand breaks of the DNA. This strategy employs a Cas9 nickase (HNH nuclease inactivated) fused to an optimized reverse transciptase domain (Anzalone et al., 2019). The prime editing guide RNA (peg sgRNA) determines the target region and also contains the information for the desired edit. A second sgRNA (nick sgRNA) triggers a nick in the unedited strand. After electroporation of the plasmids (Table 2) individual clones were expanded and the potential target site was amplified by PCR. Sanger sequencing confirmed the presence of the heterozygous mutation in exon 4 of *TBX5* (Chr 12, g.114399622, GRCh38.p13) (Fig. 1A).

The generated genome edited iPSC line shows a typical stem-cell like morphology (Fig. 1B). The pluripotent status was confirmed by immunocytochemical staining for NANOG, SOX2 and TRA1-81 (Fig. 1C) and by flow cytometry substantiating the expression of TRA1-60 and TRA1-81 on the cell surface (Fig. 1D). qRT-PCR analyses showed expression levels of endogenous pluripotency genes *OCT4, SOX2, NANOG* and *REX1* similar to other iPSC lines, but at least 1000-fold higher compared to fibroblasts from the donor (Fig. 1E and F). Cytogenetic analysis revealed a normal unaffected karyotype (Fig. 1G). The iPSC line retained its full capacity to differentiate into cells of all three germlayers as confirmed by an embryoid body-based undirected differentiation protocol (Fig. 1H) (Moretti et al., 2010).

We excluded random integration of the plasmids (Supplementary Fig. S1). Short tandem repeat analyses confirmed the genetic identity of DHMi005-A-1, the isogenic parental line DHMi005-A and the donor fibroblasts (submitted in archive with journal). Sanger sequencing of the most likely off targets for both sgRNAs used detected no genetic abnormalities (Supplementary Fig. S2). The iPSC line was free of Mycoplasma infection (Supplementary Fig. S3).

2. Materials and methods

2.1. Cell culture and gene editing

iPSCs were cultured in TeSR™E8™ (StemCell Technologies) on Matrigel-coated plates (8.7 μg/cm^2, Corning) at 37 °C and 5% CO_2 in a humidified atmosphere. To perform gene editing, cells were detached with accutase (StemCell Technologies). iPSCs (8x10^5) were electroporated with 1.5 μg pU6-pegRNA-GG-acceptor carrying the specific guide, scaffold and editing sequences (Supplementary Fig. S4), 4.5 μg pCMV-PE2 and 0.5 μg BPK1520 using the Nucleofector™II device (program B-016) (Amaxa Biosystems, Cologne) and the Human Stem Cell Nucleofector Kit-1 (Lonza, Cologne). After electroporation, iPSCs were cultured in TeSR™E8™ supplemented with 10 μM Y27632 (Calbiochem). After two days, cells were split as single cells at low density to allow growth of isolated colonies attributable to one cell in the absence of selecting agents. Individual clones were manually picked, expanded and screened by Sanger sequencing (Eurofins Genomics) of PCR fragments spanning the potential mutation site.

The correctly mutated clone was expanded and cultured in TeSR™E8™ (StemCell Technologies) on Matrigel-coated plates (8.7 μg/cm^2) at 37 °C and 5% CO_2. iPSCs were passaged every 4–6 days at a 1:6 ratio using ReLeSR (StemCell Technologies) supplemented with 10 μM Y27632.

2.2. Off target analysis

For the identification of potential off targets we used the webserver of the Center for Non-coding RNA in Technology and Health (RTH) (https://rth.dk/resources/crispr/crisproff). Sequences of 5 potential top off targets, determined for both guide RNAs, were amplified by PCR with the primers indicated in Table 2 and subsequently subjected to Sanger sequencing (Eurofins Genomics).

2.3. Genotyping and sequencing of targeted mutation

Genomic DNA from iPSCs (passage 22) was purified using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's recommendation. Genomic *TBX5* sequences were amplified as previously described (Dreßen et al., 2016) with the primers given in Table 2 and subjected to Sanger sequencing (Eurofins Genomics).

2.4. Karyotype analysis

For the in house analysis of the karyotype of iPSCs (passage 23) we applied a standard protocol (Bangs and Donlon, 2005) with the following modifications: iPSCs were incubated with Colcemid (40 ng/mL) for 2 h. Cells were detached with 0.5 mM PBS/EDTA (Gibco). Thirteen representative metaphases were evaluated and karyotyped.

2.5. Absence of random integration

The absence of random plasmid integration in genomic DNA of the genome edited iPSCs (passage 22) was verified by a PCR targeting the ampicillin resistance gene (primers in Table 2). Plasmid DNA of all three vectors used for prime editing served as positive controls.

2.6. Short tandem repeat (STR) analysis

Genomic DNA of genome edited iPSCs (passage 22), the parental

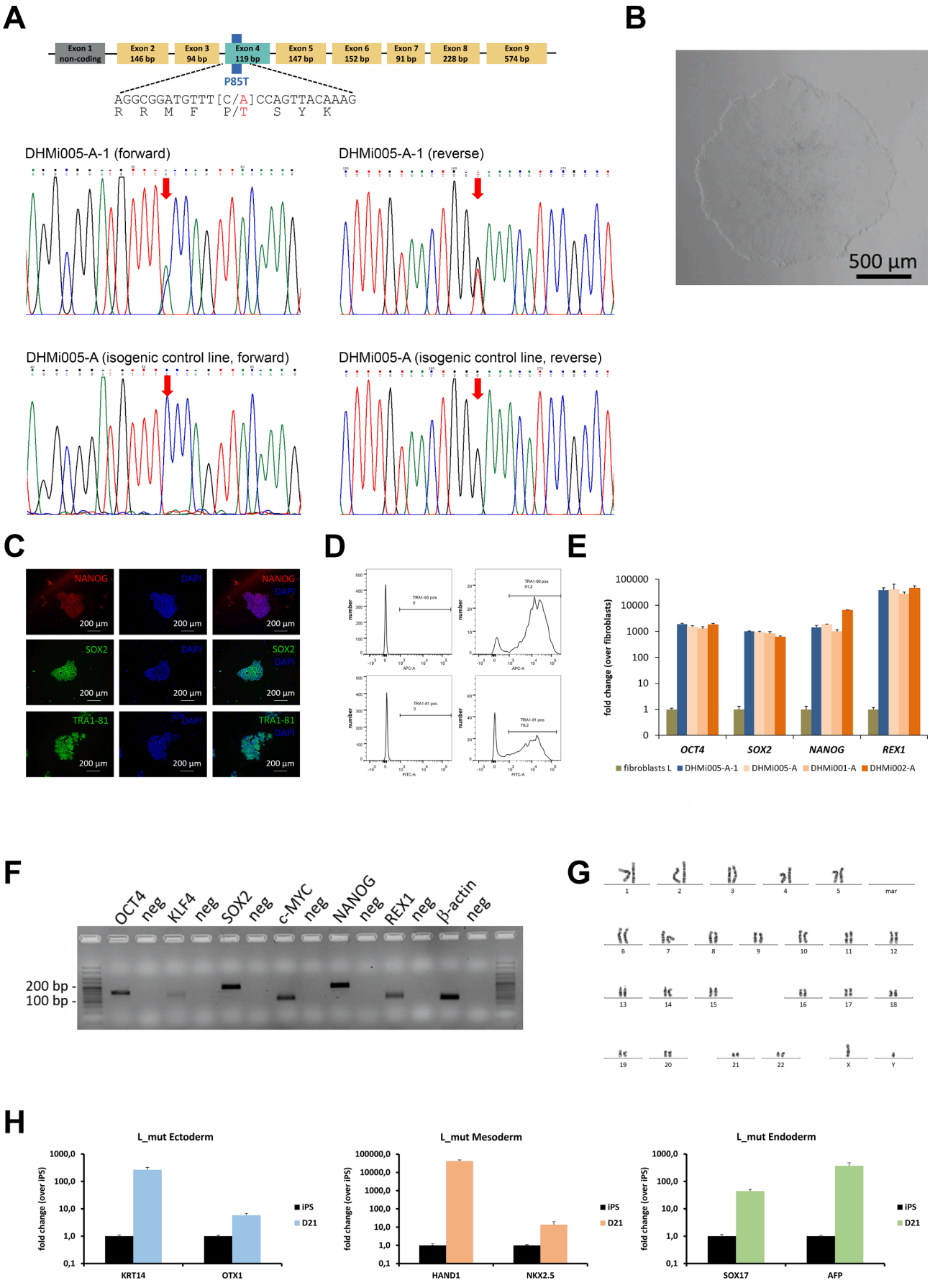

Fig. 1. Characterization of the iPSC line DHMi005-A-1 carrying a patient-specific disease mutation in exon 4 of the *TBX5* gene.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Bright field photography	Normal	Fig. 1B
Pluripotency status evidence for the described cell line	Qualitative analysis (Immunocytochemistry, RT-PCR)	Positive staining for NANOG, SOX2, and TRA1-81	Fig. 1C
		Expression of Pluripotency markers: *OCT4*, *KLF4*, *SOX2*, *c-MYC*, *NANOG*, *REX1*	Fig. 1E, Fig. 1F
	Quantitative analysis (Flow cytometry)	91.2% positive cells for cell surface marker TRA 1–60; 76.2% positive cells for cell surface marker TRA1-81	Fig. 1D
Karyotype	Karyotype (G-banding)	46XY	Fig. 1G
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site or targeted allele-specific PCR followed by Sanger sequencing	PCR across the edited site verified the heterozygous point mutation	Fig. 1A
	Transgene-specific PCR		N/A
Verification of the absence of random plasmid integration events	PCR/Southern	No random integration	Supplementary Fig. S1
Parental and modified cell line genetic identity evidence	Microsatellite PCR (mPCR)	N/A	N/A
	STR analysis	Tested 16 sites (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, AMEL, D5S818, FGA), all matched	Submitted in archive with journal
Mutagenesis/genetic modification outcome analysis	Sequencing (genomic DNA PCR or RT-PCR product)	Heterozygous point mutation	Fig. 1A
	PCR-based analyses	N/A	
	Southern Blot or WGS; western blotting (for knock-outs, KOs)	N/A	
Off-target nuclease analysis-	PCR across top 5/10 predicted top likely off-target sites	Sanger sequencing of top off target sites did not show unexpected results	Supplementary Fig. S2
Specific pathogen-free status	Mycoplasma	Mycoplasma testing by RT-PCR: Negative	Supplementary Fig. S3
Multilineage differentiation potential	Embryoid body formation	Expression of genes in embryoid bodies: *KRT14* and *OTX1* (ectoderm), *HAND1* and *NKX2.5* (mesoderm), *SOX17* and *AFP* (endoderm), qRT-PCR with *β -actin* as reference gene	Fig. 1H
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Negative	
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping	N/A	
	HLA tissue typing	N/A	

Table 2
Reagent details.

	Antibodies and stains used for immunocytochemistry/flow-cytometry		
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers (ICC)	Anti-TRA1-81, mouse monoclonal IgM	1:75	Merck Millipore, Cat# MAB4381, RRID: AB_177638
	Anti-SOX2, rabbit polyclonal IgG		Abcam, Cat# ab137385, RRID: AB_2814892
	Anti-NANOG, goat polyclonal IgG	1:250	
			Abcam, Cat# ab77095, RRID: AB_1524004
		1:75	
Secondary antibodies (ICC)	Goat Anti-Mouse IgM H&L (Alexa Fluor® 488)	1:500	Abcam, Cat# ab150121, RRID: AB_2801490
	Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488)	1:500	Abcam, Cat# ab150077, RRID: AB_2630356
	Rabbit Anti-Goat IgG H&L (Alexa Fluor® 555)	1:500	Abcam, Cat# ab150146, RRID: AB_2895679
Pluripotency Markers (FACS)	Anti-TRA1-81, mouse monoclonal IgM	1:100	Merck Millipore, Cat# MAB4381, RRID: AB_177638
	Anti TRA1-60, mouse monoclonal IgM		BD Pharmingen, Cat# 560850, RRID: AB_10565983
	Alexa Fluor® 647 coupled	1:5	
Secondary antibodies (FACS)	Goat Anti-Mouse IgM H&L (Alexa Fluor® 488)	1:2000	Abcam, Cat# ab150121, RRID: AB_2801490
Site-specific nuclease			
Nuclease information	Cas9		
Delivery method	Plasmid	Electroporation	
Selection/enrichment strategy	N/A		

Primers and Oligonucleotides used in this study

	Target	**Size of band**	**Forward/Reverse primer (5′-3′)**
Sendai-Footprinting (qRT-PCR)	SeV		5‘ GGATCACTAGGTGATATCGAGC 3‘
		181 bp	5‘ ACCAGACAAGAGTTTAAGAGATATGTATC 3‘
	ACTB (β-actin)		5‘ CCAACCGCGAGAAGATGA 3‘
		96 bp	5‘ CCAGAGGCGTACAGGGATAG 3‘
Pluripotency Markers (qRT-PCR)	*OCT4* (endogenous)		5‘ GGGATGGCGTACTGTGGG 3‘
		148 bp	5‘ GCACCAGGGGTGACGGTG 3‘

(*continued on next page*)

Table 2 (*continued*)

	Antibodies and stains used for immunocytochemistry/flow-cytometry		
	Antibody	Dilution	Company Cat # and RRID
	SOX2 (endogenous)		5‘ AGCAGACTTCACATGTCCCAG 3‘
		192 bp	5‘ ACCGGGTTTTCTCCATGCTGT 3‘
	NANOG		5‘ TGCTTTGAAGCATCCGACTGT 3‘
		193 bp	5‘ GGTTGTTTGCCTTTGGGACTG 3‘
	REX1		5‘ AGTAGTGCTCACAGTCCAGCAG 3‘
		105 bp	5‘ TGTGCCCTTCTTGAAGGTTT 3‘
Endogenous reprogramming factors (qRT-PCR)	*KLF4* (endogenous)		5′ TCTTCGTGCACCCACTTGGG 3‘
		133 bp	5‘ CTGCTCAGCACTTCCTCAAG 3‘
	C-MYC (endogenous)		5‘ CACCAGCAGCGACTCTGA 3‘
		101 bp	5‘ GATCCAGACTCTGACCTTTTGC 3‘
House-keeping gene (qRT-PCR)	*ACTB (β -actin)*	96 bp	5‘ CCAACCGCGAGAAGATGA 3‘
			5‘ CCAGAGGCGTACAGGGATAG 3‘
Differentiation markers			
Ectoderm			
	KRT14	85 bp	5′ CACCTCTCCTCCTCCCAGTT 3′
			5′ ATGACCTTGGTGCGGATTT 3′
	OTX1	125 bp	5′ GATCAACCTGCCGGAGTCTA 3′
			5′ CGCACTGGAGAGGACTTCTT 3′
Mesoderm			
	HAND1	72 bp	5′ AACTCAAGAAGGCGGATGG 3′
			5′ GGAGGAAAACCTTCGTGCT 3′
	NKX2.5	102 bp	5′ TTCTATCCACGTGCCTACAGC 3′
			5′ CTGTCTTCTCCAGCTCCACC 3′
Endoderm			
	SOX17	81 bp	5′ ACGCCGAGTTGAGCAAGA 3′
			5′ TCTGCCTCCTCCACGAAG 3′
	AFP	90 bp	5′ GTGCCAAGCTCAGGGTGTAG 3′
			5′ CAGCCTCAAGTTGTTCCTCTG 3′
Genotyping (desired allele/transgene presence detection)	*TBX5*		5′ TGGATGGAGGCTGCCTTAAAA 3′
		273 bp	5′ GTTCACTGATACACTTTTCAAC 3′
Targeted mutation analysis/sequencing	*TBX5*		5′ TGGATGGAGGCTGCCTTAAAA 3′
		273 bp	5′ GTTCACTGATACACTTTTCAAC 3′
Potential random integration-detecting PCRs	Vector backbone Amp^R		5′ CAGTGCTGCAATGATACCGCGA 3′
		477 bp	5′ ACTCGGTCGCCGCATACACTAT 3′
gRNA oligonucleotide sequences	peg sgRNA		5′ GTCACCTTCACTTTGTAACT 3′
	nick sgRNA		5′ TGGAGGCTGCCTTAAAATAC 3′
Genomic target sequence(s)	peg sgRNA		5′ GTCACCTTCACTTTGTAACT GGG 3′ (+) (Chr. 12 at position 114399617)
	nick sgRNA		5′ TGGAGGCTGCCTTAAAATAC TGG 3′ (-) (Chr. 12 at position 114399682)
On target	TBX5		5′ TGGATGGAGGCTGCCTTAAAA 3′
		273 bp	5′ GTTCACTGATACACTTTTCAAC 3′
Off-target sites	Off target 1		5′ AGAGCTGGAGGGTCACACACTG 3′
peg sgRNA		379 bp	5′ CAAGGGCAAGGGTCCTGAGAGT 3′
	Off target 2		5′ GGCAGCCTCTAAAATGCCCCAC 3′
		275 bp	5′ TGGAGGAAAGCAGGCTCCAGTT 3′
	Off target 3		5′ ATGCTCCCGTTTGCAAAGCCAT 3′
		312 bp	5′ TGGTTTTGTTGGTTGCCCCATCA 3′
	Off target 4		5′ ACAGAGTACAAACTGTGAGGGGT 3′
		359 bp	5′ TGGTTCCCGACTAACGATGGCT 3′
	Off target 5		5′ CATCTCGGGCCGTGGACAAATG 3′
		278 bp	5′ GTGGGTAGGACAACTGGGCTGA 3′
Off target sites	Off target 1		5′ CTCTCCCTCCCCTCCTCCATCT 3′
nick sgRNA		224 bp	5′ GTGCACACACAGTGAACCCGAA 3′
	Off target 2		5′ CCGGGATGTCTTCAGGGGCAAT 3′
		278 bp	5′ TCACATTTGTGTTGATGCTGGTGT 3′
	Off target 3		5′ TGCACATGGCCTCTCTCTGGAA 3′
		219 bp	5′ AAGAGCAAGGAGTCTTCCGGGG 3′
	Off target 4		5′ CCCAACAAGAATGAGTCCCTGGC 3′
		363 bp	5′ CCTTCAAGCCACAAGCATCCTGT 3′
	Off target 5		5′ CAGTTCCCTACCTGCCCATGGT 3′
		374 bp	5′ ACTTTGGGTCCTGAAGAAGTGA 3′
ODNs/plasmids/RNA templates used as templates for HDR-mediated site-directed mutagenesis. Backbone modifications in utilized ODNS have to be noted using standard nomenclature.			pCMV-PE2 (Addgene #132775) (RRID: Addgene_132775) pU6-pegRNA-GG-acceptor (Addgene #132777) (RRID: Addgene_132777) BPK1520 (Addgene #65777) (RRID: Addgene_65777)

isogenic iPSC line (passage 28) and fibroblasts of the donor (passage 3) were sent to Eurofins Genomics. Genetic characteristics were determined by PCR-single-locus-technology. Sixteen independent markers (Table 1) were investigated together with appropriate positive and negative controls.

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.

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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.2022.102691.

References

Anzalone, A.V., Randolph, P.B., Davis, J.R., Sousa, A.A., Koblan, L.W., Levy, J.M., Chen, P.J., Wilson, C., Newby, G.A., Raguram, A., Liu, D.R., 2019. Search-and-replace genome editing without double-strand breaks or donor DNA. Nature 576 (7785), 149–157. https://doi.org/10.1038/s41586-019-1711-4.

Bangs, C.D., Donlon, T.A., 2005. Metaphase chromosome preparation from cultured peripheral blood cells. Curr. Protoc. Human Genet. Chapter 4 (1).

Dreßen, M., Lahm, H., Lahm, A., Wolf, K., Doppler, S., Deutsch, M.-A., Cleuziou, J., Pabst von Ohain, J., Schön, P., Ewert, P., Malcic, I., Lange, R., Krane, M., 2016. A novel de novo *TBX5* mutation in a patient with Holt-Oram syndrome leading to a dramatically reduced biological function. Mol. Genet. Genomic Med. 4 (5), 557–567. https://doi.org/10.1002/mgg3.234.

Moretti, A., Bellin, M., Welling, A., Jung, C.B., Lam, J.T., Bott-Flügel, L., Dorn, T., Goedel, A., Höhnke, C., Hofmann, F., Seyfarth, M., Sinnecker, D., Schömig, A., Laugwitz, K.-L., 2010. Patient-specific induced pluripotent stem-cell models for long-QT syndrome. N. Engl. J. Med. 363 (15), 1397–1409. https://doi.org/10.1056/NEJMoa0908679.