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Lab Resource: Genetically-Modified Single Cell Line

Generation of miR-15a/16-1 cluster-deficient human induced pluripotent stem cell line (DMBi001-A-2) using CRISPR/Cas9 gene editing

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ABSTRACT

miR-15a/16-1 cluster, composed of *MIR15A* and *MIR16-1* genes located in close proximity on chromosome 13 was described to regulate post-natal cell cycle withdrawal of cardiomyocytes in mice. In humans, on the other hand, the level of miR-15a-5p and miR-16-p was negatively associated with the severity of cardiac hypertrophy. Therefore, to better understand the role of these microRNAs in human cardiomyocytes in regard to their proliferative potential and hypertrophic growth, we generated hiPSC line with complete deletion of miR-15a/16-1 cluster using CRISPR/Cas9 gene editing. Obtained cells demonstrate expression of pluripotency markers, differentiation capacity into all three germ layers and normal karyotype.

Resource Table:

Unique stem cell line identifier	DMBi001-A-2 (https://hpscreg.eu/user/cellline/edit/DMBi001-A-2)
Alternative name(s) of stem cell line	*N/A*
Institution	Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland
Contact information of the reported cell line distributor	Prof. Józef Dulak PhD, mail: jozef.dulak@uj.edu.pl Jacek Stępniewski, PhD, mail: jacek.stepniewski@uj.edu.pl
Type of cell line	hiPSC
Origin	human
Additional origin info *(applicable for human ESC or iPSC)*	Age: 58 Sex: male Ethnicity: caucasian
Cell Source	Healthy hiPSC line DMBi001-A (https://hpscreg.eu/cell-line/DMBi001-A)
Method of reprogramming	Sendai virus
Clonality	clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	RT-PCR
Cell culture system used	Geltrex™, Essential 8 medium, ROCK inhibitor Y27623 (10 μM) added with every replating; cells were passaged with 0,5 mM EDTA at a ratio of 1:10; cells were cultured in 37C, 5 % CO_2, 20 % O_2
Type of Genetic Modification	CRISPR/Cas9-mediated deletion
Associated disease	*N/A*
Gene/locus	*MIR15A* and *MIR16-1*, 13q14.2 (Gene ID: 406948 and 406950, respectively)
Method of modification/site-specific nuclease used	CRISPR/Cas9
Site-specific nuclease (SSN) delivery method	Plasmid transfection
All genetic material introduced into the cells	Cas9 plasmids pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene #62988)
Analysis of the nuclease-targeted allele status	Sequencing of the targeted allele
Method of the off-target nuclease activity surveillance	Surveyor nuclease assay
Name of transgene	N/A
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	Positive (puromycin)
Inducible/constitutive system details	N/A
Date archived/stock date	25.07.2022
Cell line repository/bank	Human Pluripotent Stem Cell Registry (hPSC^{reg}) https://hpscreg.eu/user/cellline/edit/DMBi001-A-2
Ethical/GMO work approvals	Jagiellonian University Bioethical Committee, approval No.

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Abbreviations: CRISPR/Cas9, Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR-associated protein 9; hiPSC, human induced pluripotent stem cells; miR-15a, microRNA-15a; miR-16-1, microRNA-16-1; sgRNA, single guide RNA.

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(*continued*)

	122.6120.303.2016 Agreement of the Ministry of the Environment for the use of GMO/GMM (decision 41/2016)
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	pSpCas9(BB)-2A-Puro (PX459) V2.0 was a gift from Feng Zhang (Addgene plasmid #62988; RRID:Addgene_62988)

1. Resource utility

A human induced pluripotent stem cell (hiPSC) line with CRISPR/Cas9-mediated complete deletion of *MIR15A* and *MIR16-1* on chromosome 13 was generated to study the anti-proliferative activity of miR-15a-5p and miR-16-5p in human cardiomyocytes upon cardiac differentiation.

2. Resource details

Prenatal heart development mainly depends on proliferation of cardiomyocytes, whereas shortly after birth proliferation is stopped and heart starts hypertrophic growth (Li et al., 1996). Both stages are tightly regulated by miRNAs. Particularly, expression of the members of miR-15 family, including miR-15a-5p and miR-16-5p, elevates by postnatal day10 in mice which correlates with cell cycle withdrawal in cardiomyocytes (Porrello et al., 2011). *MIR15A* and *MIR16-1* genes encoding miR-15a-5p and miR-16-5p, respectively, are located on chromosome 13 within one cluster. Interestingly, recent study indicated that expression of these miRNAs negatively correlates with the severity of hypertrophic cardiomyopathy in humans (Guo et al., 2020). Thus, the precise role of miR-15a-5p and miR-16-5p regarding human cardiomyocytes proliferation and hypertrophic growth warrants further studies.

Taking this into consideration, we decided to generate hiPSC line with complete deletion of miR-15a/16-1 cluster encompassing *MIR15A* and *MIR16-1* genes located in close proximity on chromosome 13. For this purpose, CRISPR/Cas9 gene editing was applied in previously described healthy hiPSC line (Stępniewski et al., 2020). Particularly, two sgRNAs targeting 3′ downstream region of *MIR15A* and 2 sgRNAs targeting 5′ upstream region of *MIR16-1* were designed, proper oligo DNA sequences cloned into Cas9-expressing plasmid (pSpCas9(BB)-2A-Puro V2.0, Addgene #62988) and introduced into hiPSC using nucleofection method. Upon selection with puromycin, DNA was isolated from the cells to evaluate the cleavage activity within targeted locus. This analysis revealed the optimal combination of sgRNA1 (5′ TGTGCTGCTACTTTACTCCA 3′) binding to 3′ downstream region of *MIR15A* and sgRNA2 (5′TAAAACACAACTGTAGAGTA 3′) (Fig. 1A) binding to 5′ upstream site of *MIR16-1*, thus hiPSC treated with these sgRNAs were seeded in low density to obtain single-cell derived clones. Eventually, genotyping of obtained clones revealed one with complete deletion of miR-15a/16-1 cluster on both alleles (Fig. 1B). The presence of the mutation was then confirmed by Sanger sequencing (Fig. 1A, Supplementary File 1) which additionally indicated that the same modified sequence is present on both alleles.

After expansion, the DMBi001-A-2 hiPSC line demonstrated typical hiPSC morphology (Fig. 1C) while the immunofluorescent analysis confirmed the presence of pluripotency markers (OCT4, NANOG, SSEA4, TRA-1–60 and TRA-1–81) in these cells. (Fig. 1D). RT-qPCR analysis additionally revealed that expression of *NANOG*, *SOX2*, *SALL4* and *TERT* in DMBi001-A-2 hiPSC was similar to its parental, previously described DMBi00-1-A line (Stępniewski et al., 2020) (Fig. 1E). Upon spontaneous differentiation via embryoid bodies (EB), cells originating from three germ layers were detected by immunofluorescent analysis [GATA4 as endo and mesodermal marker; alpha fetoprotein (AFP) as endodermal marker and neurofilament heavy chain (NFH) as ectodermal marker] (Fig. 1F) as well as RT-PCR (*NESTIN* and *TUBB3* – ectoderm, *NKX2.5* and *MEF2C* – mesoderm and *FOXA2* – endoderm) (Fig. 1G). Generated line demonstrated healthy karyotype (Fig. 1H) and lack of mycoplasma contamination (Supplementary Fig. 1). Importantly, qRT-PCR-based analysis confirmed lack of miR-15a-5p and potently decreased miR-16-5p expression in spontaneously differentiated DMBi001-A-2 cells (Fig. 1I) (confirmation of the complete lack of miR-16-5p expression was not possible as *MIR16-2* gene located on chromosome 3 encodes exactly the same miR-16-5p sequence as deleted *MIR16-1*).

3. Materials and methods

3.1. Generation and culture of DMBi001-A-2 hiPSC

To obtain DMBi001-A-2 hiPSC line, healthy hiPSC were nucleofected with pSpCas9(BB)-2A-Puro (PX459) V2.0 plasmids (Addgene #62988) encoding Cas9 as well as sgRNA1 (5′ TGTGCTGCTACTTTACTCCA 3′) and sgRNA2 (5′TAAAACACAACTGTAGAGTA 3′), using Human Stem Cell Nucleofector™ Kit 1 and Amaxa Nucleofector 2B (program B16, both Lonza). 24 h after nucleofection, cells were selected using 0,3 μg/ml puromycin (Sigma-Aldrich) which was added to the culture medium for 24 h. Subsequently, 500 cells were seeded on 6 cm culture dish to obtain single cell-derived clones, which were picked, subjected to DNA isolation (Genomic Mini kit, A&A Biotechnologies) and genotyped using PCR-based method with primers recognizing miR-15a/16-1 cluster-flanking sites (Fig. 1A, Table 2, expected band length for wild type locus – 855 bp and for modified locus – approximately 628 bp). One clone which demonstrated shorter band indicating complete deletion of *MIR15A* and *MIR16-1* in both alleles was further expanded and subjected to characterization. sgRNAs used in this study were designed using CHOPCHOP software (https://chopchop.cbu.uib.no/).

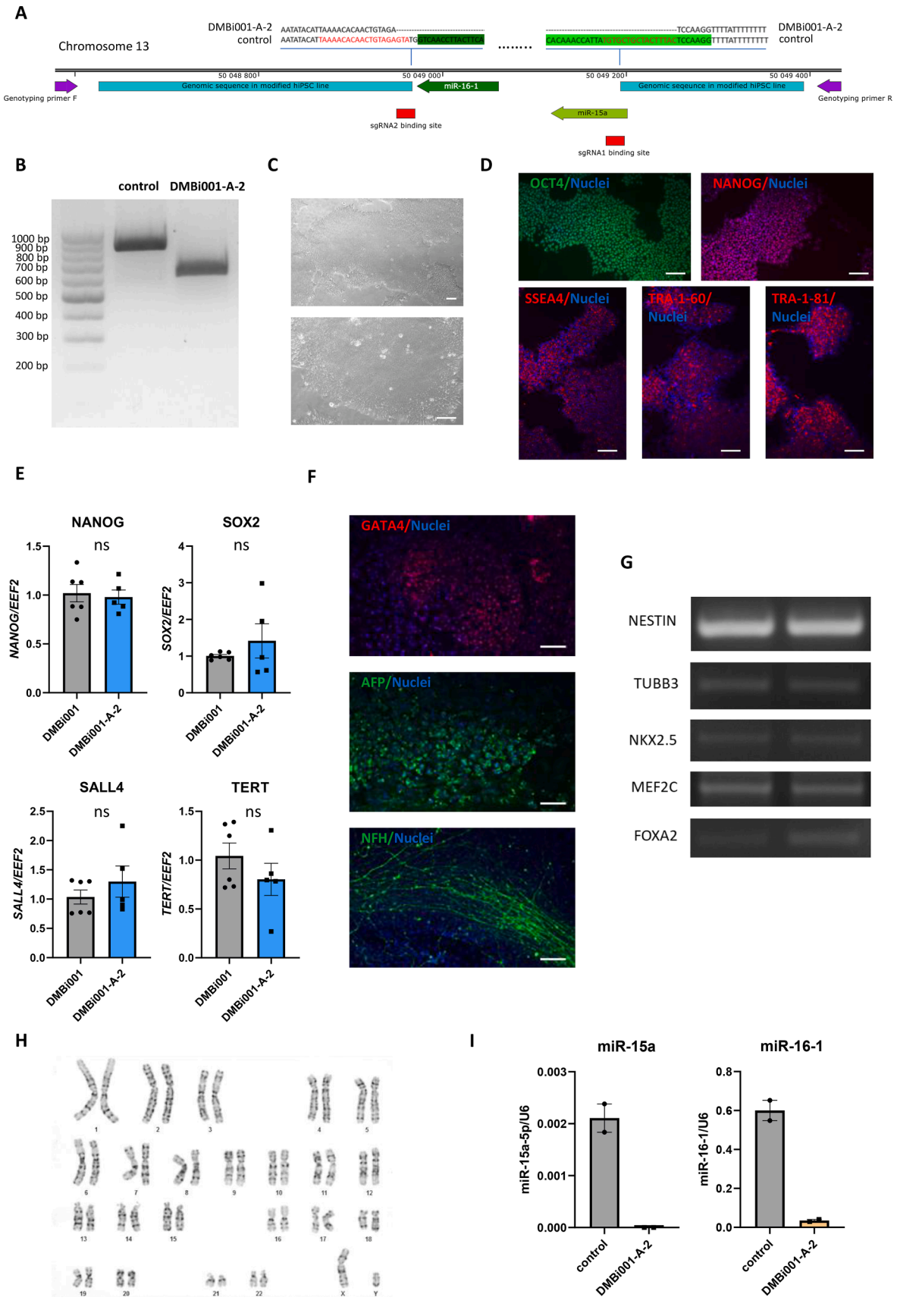
DMBi001-A-2 hiPSC hiPSC were cultured on Geltrex™ (ThermoFisher Scientific)-coated plates in Essential 8 medium (ThermoFisher Scientific) which was refreshed on daily basis. Passages (1:10, every 4 – 5 days) were performed using 0.5 mM EDTA with ROCK inhibitor (Y27632, 10 μM, Abcam) added to the cell culture for 24 h after each passage. Cell culture was performed under standard conditions (37C, 5 % CO_2, 20 % O_2).

3.2. In vitro spontaneous differentiation

DMBi001-A-2 hiPSC were harvested, resuspended in Essential 6 medium (Thermofisher Scientific) with ROCK inhibitor and seeded onto non-adherent U-shaped 96-well plate (3000 cells per one EB). Medium was refreshed after 48 h and after additional 72 h, EBs were transferred onto Geltrex-covered 48-well plate (one EB per one well). On day 14 of differentiation, obtained cells were subjected to immunofluorescent and RT-PCR-based analysis of the expression of markers of three germ layers.

3.3. Immunofluorescent analysis (IF)

For immunofluorescent analysis, cells were fixed with 4 % paraformaldehyde for 15 min in room temperature (RTemp) and permeabilized with 0.1 % Triton X-100 in PBS for 30 min, RTemp. Subsequently, cells were washed three times with PBS and blocked with 5 % bovine serum albumin for 60 min, RTemp. Primary antibodies were diluted in blocking solution (as indicated in Table 1) and incubated overnight in 4 °C. Then, cells were washed five times in PBS and secondary antibodies (as indicated in Table 1) were added for 60 min, RTemp. Nuclei were stained with 1 μg/mL Hoechst33342 (Sigma-Aldrich). Pictures were taken under fluorescent microscope Nikon Eclipse TS100.



(caption on next page)

Fig. 1. Generation and characterization of DMBi001-A-2 hiPSC line with complete deletion of miR-15a/miR-16-1 cluster on chromosome 13. A. Schematic representation of binding sites for applied sgRNAs (in red) and primers used for detecting introduced mutations within chromosome 13 (in purple) as well as the representation of genomic fragment left after deletion of *MIR15A* and *MIR16-1* (in blue). Aligned sequences from control and DMBi001-A-2 hiPSC lines showing deletion breakpoints are presented above the scheme (red font – sgRNA template sequences, highlighted in dark green – fragment of *MIR16-1* gene, highlighted in light green – fragment of *MIR15A* gene; for the exact sequence present in DMBi001-A-2 hiPSC line see Supplementary File 1). B. PCR-based genotyping of DMBi001-A-2 indicating complete deletion of miR-15a/16-1 cluster in both alleles (shorter band of 628 bp). DMBi001-A parental line served as a control (longer band of 855 bp derived from non-modified locus). C. Brightfield morphology DMBi001-A-2 line (scale bars: 50 μm, magnification: top: 100x, bottom: 200x). D. Immunofluorescent analysis of pluripotency markers: OCT4 (green), NANOG, SSEA4, TRA-1–60 and TRA-1–81 (red) in DMBi001-A-2 line. Nuclei stained with Hoechst33342 (blue). Scale bars: 50 μm. E. qRT-PCR analysis of the expression of pluripotency markers: *NANOG*, *SOX2*, *SALL4* and *TERT* in DMBi001-A-2 hiPSC in comparison to the parental DMBi001-A hiPSC line. N = 3, RNA isolated from 3 independent passages, analysis performed in technical duplicates. ns – statistically nonsignificant, *t*-test. F. Immunofluorescent analysis of three germ layers markers in spontaneously differentiated DMBi001-A-2 hiPSC. GATA4 (red) – endo and mesodermal marker, AFP (green) – endodermal marker and NFH (green) - ectodermal marker. Nuclei were stained with Hoechst33342 (blue). Scale bars: 50 μm. G. RT-PCR-based analysis of three germ layers markers in spontaneously differentiated DMBi001-A-2 hiPSC. *NESTIN* and *TUBB3* – ectodermal markers, *NKX2.5* and *MEF2C* – mesodermal markers and *FOXA2* – endodermal marker. H. Karyotype of DMBi001-A-2 hiPSC line. I. qRT-PCR analysis of miR-15a-5p (left) and miR-16-5p (right) expression in spontaneously differentiated DMBi001-A-2 and DMBi001-A (control) hiPSC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Characterization and validation.

Classification (optional *italicized*)	Test	Result	Data
Morphology	Photography	*normal*	Fig. 1, panel C
Pluripotency status evidence for the described cell line	Qualitative analysis *(i.e. Immunocytochemistry, western blotting) [mandatory]*	Assessed staining of pluripotency markers: OCT4, NANOG presence in the nuclei, SSEA4, TRA-1–60 and TRA-1–81 present on the cytoplasmic membrane	Fig. 1, panel D
	Quantitative analysis *(i.e. Flow cytometry, RT-qPCR)*	qRT-PCR analysis of pluripotency markers: *NANOG*, *SOX2*, *SALL4* and *TERT* in comparison to previously described parental DMBi001-A hiPSC line (Stępniewski et al., 2020)	Fig. 1, panel E
Karyotype	Karyotype (G-banding)	*46XY, Resolution 450–500*	Fig. 1, panel H
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site or targeted allele-specific PCR	Deletion of *MIR15A* and *MIR16-1* cluster located on chromosome 13 (50048968–50049194 bases of the chromosome 13 annotated NC_000013.11 are deleted), homozygous	Fig. 1, panel A, Supplementary File 1
	Transgene-specific PCR	*N/A*	*N/A*
Verification of the absence of random plasmid integration events	PCR	PCR detection using primers recognizing Cas9 coding sequence. Integrity of DNA was confirmed using primers recognizing intron 2 of *HBB* gene on chromosome 11	Supplementary Fig. 2
Parental and modified cell line genetic identity evidence	STR analysis	DNA Profiling (AMEL, D5S818, D13S317, D7S820, D16S539, vWA, Th01, TPOX, CSF1PO, D21S11), matched with paternal cell line	Supplementary File 2
Mutagenesis/genetic modification outcome analysis	Sequencing (genomic DNA PCR product)	Deletion of *MIR15A* and *MIR16-1* cluster located on chromosome 13 (50048968–50049194 bases of the chromosome 13 annotated NC_000013.11 are deleted), homozygous. The same mutation in both alleles	Fig. 1, panel A, Supplementary File 1
	PCR-based analyses	Detection of homozygously excised PCR product	Fig. 1, panel C
	qRT-PCR	Lack of miR-15a-5p and decreased detection of miR-16-1-5p expression in DMBi001-A-2 hiPSC-derived spontaneously (via EB) differentiated cells	Fig. 1, panel I
Off-target nuclease analysis	Sanger sequencing (Genomed)	Sequences of 5 selected off-target sites for sgRNA1 and sgRNA2 - lack of mutations introduced by unspecific activity of sgRNAs	Supplementary File 3
Specific pathogen-free status	PCR	Negative for *Mycoplasma* spp.	Supplementary Fig. 1
Multilineage differentiation potential	Embryoid body formation	Demonstrated ability to differentiate into derivatives of 3 germ layers: ectoderm (neurofilament H – immunofluorescent staining; *NESTIN* and *TUBB3* – RT-PCR); mesoderm (GATA4 - immunofluorescent staining; *NKX2.5* and *MEF2C* – RT-PCR); endoderm (Alpha Fetoprotein – immunofluorescent staining, *FOXA2* – RT-PCR)	Fig. 1, panel F and G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	*N/A*	N/A
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping	*N/A*	N/A
	HLA tissue typing	*N/A*	N/A

3.4. RT-PCR, PCR and quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using fenozol reagent (A&A Biotechnology) and chloroform. Reverse transcription (RT) was performed using oligodT primers (10 μM) and RevertAid Reverse Transcriptase (Thermofisher Scientific). Genomic DNA was isolated using Genomic Mini kit (A&A Biotechnologies). PCR, both for genotyping of the hiPSC clones and upon RT was performed using specific primers (indicated in Table 2) and KAPA2G Fast Genotyping Mix (Sigma-Aldrich). The amplified products were electrophoretically separated in 2 % agarose gel.

Expression of pluripotency markers was performed using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) and primers recognizing *NANOG*, *SOX2*, *SALL4* and *TERT* whereas *EEF2* served as a reference gene (Table 2). cDNA from DMBi001-A-2 hiPSC and parental, previously described, DBMi001-A line (Stępniewski et al., 2020) obtained from three independent passages was applied for the analysis.

miR-15a-5p and miR-16-5p expression was analysed using RNA isolated from spontaneously differentiated DMBi001-A-2 hiPSC as well as their parental DMBi001-A line which served as a control. RT was performed using LNA miRCURRY RT kit which was followed by quantitative PCR with the application of miRCURY LNA SYBR® Green PCR Kit, both according to the manufacturer's protocol (Qiagen). LNA primers recognizing U6 (as a reference), miR-15a-5p and miR-16-5p were used (Qiagen, Table 2).

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Primary antibodies -pluripotency markers (IF or FC)	goat anti-OCT3/4mouse anti-NANOG mouse anti-SSEA4 mouse anti-TRA1-60 mouse anti-TRA1-81	1:200 IF 1:100 IF 1:100 IF 1:100 IF 1:100 IF	Santa Cruz Biotechnology Cat# sc-8628, RRID:AB_653551 Santa Cruz Biotechnology Cat# sc-293121, RRID:AB_2665475 Santa Cruz Biotechnology Cat# sc-21704, RRID:AB_628289 Millipore Cat#MAB4360, RRID:AB_2119183 Millipore Cat# MAB4381, RRID:AB_177638
Primary antibodies -germ layers' markers (IF)	goat anti-AFP mouse anti-GATA4 rabbit anti-NFH	1:100 1:200 1:500	Santa Cruz Biotechnology Cat# sc-8108, RRID:AB_633815 Santa Cruz Biotechnology Cat# sc-25310, RRID:AB_627667 Abcam Cat# ab8135, RRID: AB_306298
Secondary antibodies	rabbit anti-goat IgG AF488 goat anti-mouse IgG AF568 goat anti-rabbit IgG AF488	1:400 IF 1:400 IF 1:400 IF	Thermo Fisher Scientific Cat# #A-11078, RRID:AB_2534122 Thermo Fisher Scientific Cat# A-11004, RRID:AB_2534072 Thermo Fisher Scientific Cat# A-11034, RRID:AB_2576217
Nuclear stain	Hoechst33342 (IF and FC)	1 μg/ml	Sigma-Aldrich
Site-specific nuclease			
Nuclease information	Cas9		pSpCas9(BB)-2A-Puro (PX459) V2.0 was a gift from Feng Zhang (Addgene plasmid #62988; https://n2t.net/addgene:62988; RRID:Addgene_62988)
Delivery method	Plasmid nucleofection		
Selection/enrichment strategy	Puromycin 0,3 μg/ml, 24 h after nucleofection, for 24 h		
Primers and Oligonucleotides used in this study			
	Target		**Forward/Reverse primer (5′-3′)**
qRT-PCR analysis of the pluripotency markers	*NANOG*		AGCAGATGCAAGAACTCTCCAA/ TAAAGGCTGGGGTAGGTAGGTG Fig. 1, panel E
	SOX2		GCGGAAAACCAAGACGCTC/ TCATGTGCGCGTAACTGTCC Fig. 1, panel E
	SALL4		TGTGGCGGAGAGGGCAAATA/ GTGGCTTCATCCTCACTCGC Fig. 1, panel E
	TERT		GCATCAGGGGCAAGTCCTAC/ TACTCAGGGACACCTCGGAC Fig. 1, panel E
	EEF2		TCAGCACACTGGATAGAGG/ GACATCACCAAGGGTGTGCA Fig. 1, panel E, reference gene
RT-PCR analysis of the markers of ectoderm, mesoderm and endoderm in spontaneously differentiated DMBi001-A-2 hiPSC	*NESTIN*		GCGGGCTACTGAAAAGTTCC/ CCAGCTTGGGGTCCTGAAAG Fig. 1, panel G
	TUBB3		AGGGCATCTCTTGAGAACAAA/ GCCTCGTTGTAGTAGACGCTG Fig. 1, panel G
	NKX2.5		GGACCCTAGAGCCGAAAAGAAAG/ TCTTGACCTGCGTGGACGTGA Fig. 1, panel G
	MEF2C		ATTCCGTAGGTCACAGCCCT/ CGAAGTTGGGAGGTGGAACA Fig. 1, panel G
	FOXA2		ATTGCTGGTCGTTTGTTGTG/ TACGTGTTCATGCCGTTCAT Fig. 1, panel G
Genotyping (desired deletion presence detection) (PCR)	*MIR15A – MIR16-1* cluster on chromosome 13		GTCTTCTAAGCTCTGTTCAAAATGC/ CTTTGTGTTTCCTAACCTATAGCAC
Targeted mutation analysis/sequencing	Sequencing data from both alleles of *MIR15A – MIR16-1* cluster on chromosome 13		Sanger sequencing Supplementary File 1
qRT-PCR analysis of miR-15a-5p and miR-16-5p expression	LNA miRCURRY RT kit followed by quantitative PCR with the application of miRCURY LNA SYBR® Green PCR Kit (Qiagen).		hsa-miR-15a-5p miRCURY LNA miRNA PCR Assay (cat no: YP00204066, Qiagen) hsa-miR-16-5p miRCURY LNA miRNA PCR Assay (cat no: YP00205702, Qiagen) U6 snRNA(hsa, mmu) miRCURY LNA miRNA PCR Assay (cat no: YP00203907, Qiagen) Fig. 1, panel I

(*continued on next page*)

Table 2 (*continued*)

Antibodies and stains used for immunocytochemistry/flow-cytometry		
	Antibody	Dilution Company Cat # and RRID
Potential random integration detection	*Cas9* (PCR) *HBB* intron 2 (chromosome 11) – to confirm integrity of DNA (PCR) (De et al., 2018)	CATCGAGCAGATCAGCGAGT/ CGATCCGTGTCTCGTACAGG Supplementary Fig. 2 CTATGGGACGCTTGATGT/ GCAATCATTCGTCTGTT Supplementary Fig. 2
Specific pathogen-free status(PCR)	*Mycoplasma* spp.	ACTCCTACGGGAGGCAGCAGTA/ TGCACCATCTGTCACTCTGTTAACCTC Supplementary Fig. 1
sgRNA1 sgRNA2	5′TGTGCTGCTACTTTACTC CA3′ 5′TAAAACACAACTGTAGAGTA3′	50,049,179 – 50,049,198 (reference ID NG_000013.11) 50,049,199 – 50,049,201 (reference ID NG_000013.11) – PAM sequence (AGG) 50,048,951–50,048,970 (reference ID NG_000013.11) 50,048,971 – 50,048,973 (reference ID NG_000013.11) – PAM sequence (TGG)
5 selected off-target sites for sgRNA1	Off-target 1, Chromosome 6: 86,069,791 Off-target 2, chromosome 7: 96,685,411 Off-target 3, chromosome 17: 45,104,465 Off-target 4, chromosome 10: 130,336,639 Off-target 5, chromosome 1: 24,837,911	GGAAGGGATACTCTGAAGG/ CCACTGGTTCATAGTTGGCCAG CTTGGCTCATTATGATAGTCCTC/ GTGTGTTAAGTTTCAGAACAGCCT GAGATCCAGTGCATTGAACAC/
5 selected off-target sites for sgRNA 2	Off-target 1, chromosome 1: 205,433,066 Off-target 2, chromosome 12: 48,603,034 Off-target 3, chromosome 4: 115,894,849 Off-target 4, chromosome 1: 56,528,898 Off-target 5, chromosome 5: 18,400,625	GCATCCAACTGCCAGTAGC CAGCTTTCACATCACCCATGTAG/ GCCAGGTTAGGATGGAAGG CCTTGGTCCCTTCATGGTAGTC/ CCAGCAACATAACAAAAGGTAG GAAAACAATGTGGCAAGGCTC/ CCTGTGACAGAAGAGGCTTCCTATC CAAGAGTAGGAACCCACG/ GTCTTACATGGACAGTGTTGTG GCTCTAGCATCTTGAACTC/ CTTACACTTATTGATGGACCAC GCCTAGTAGGACTTCTAAGAGAAAG/ GTAGAGAGTATCTCTTCCAAGAGC GTAACCAAGTATCTTGTGCTTGTC/ TTCTTGATGTTCTTTGCACCCT Supplementary File 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.	*N/A*	*N/A*

3.5. Mutation validation

Deletion of miR-15a/16-1 cluster was confirmed by Sanger sequencing of the targeted locus (Genomed S.A.) after PCR amplification with specific primers (Table 2) and DNA recovery from gel using Zymoclean™ Gel DNA Recovery Kit (Zymo Research).

3.6. Lines identity validation

STR analysis of DMBi001-A-2 hiPSC was performed by Human Genome Variation Research Group (Małopolska Centre of Biotechnology, Krakow, Poland) and the obtained profile was compared to the parental line (DMBi001-A).

3.7. Karyotype

Karyotype analysis was performed in Kariogen cytogenic laboratory (Kraków, Poland) using the G-banding method. 10 mitoses were analyzed.

3.8. Mycoplasma

Mycoplasma contamination was verified by PCR for *Mycoplasma spp*. DNA (Table 2).

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.

Acknowledgments

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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.2023.103046.

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