Generation and genetic repair of 2 iPSC clones from a patient bearing a heterozygous c.1120del18 mutation in the ACVRL1 gene leading to Hereditary Hemorrhagic Telangiectasia (HHT) type 2

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Lab Resource: Multiple Stem Cell Lines

Generation and genetic repair of 2 iPSC clones from a patient bearing a heterozygous c.1120del18 mutation in the *ACVRL1* gene leading to Hereditary Hemorrhagic Telangiectasia (HHT) type 2

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ABSTRACT

Fibroblasts from a patient carrying a heterozygous 18bp deletion in exon 8 of the *ACVRL1* gene (c.1120del18) were reprogrammed using episomal vectors. The in-frame deletion in ACVRL1 causes the loss of 6 amino acids of the protein, which is associated with Hereditary Hemorrhagic Telangiectasia (HHT) type 2 (Letteboer et al., 2005). CRISPR-Cas9 editing was used to genetically correct the mutation in the induced pluripotent stem cells (iPSCs). The top5-predicted off-target sites were not altered. Patient and isogenic iPSCs showed high pluripotent marker expression, *in vitro* differentiation capacity into all three germ layers and displayed a normal karyotype. The obtained isogenic pairs will enable proper *in vitro* disease modelling of HHT (Roman and Hinck, 2017).

Resource Table:

Unique stem cell lines identifier	LUMCi030-A LUMCi030-B LUMCi030-A-1 LUMCi030-B-1
Alternative names of stem cell lines	LUMC0110iALK04 LUMC0110iALK10 iso03LUMC0110iALK04 iso01LUMC0110iALK10
Institution	Leiden University Medical Center, Leiden, the Netherlands
Contact information of distributor	V. Orlova, PhD, HYPERLINK "mailto: V.Orlova@lumc.nl
Type of cell lines	iPSC
Origin	Human
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogramming	Episomal vectors: OCT3/4, SOX2, KLF4, L-MYC, LIN28
Multiline rationale	Gene corrected clones
Gene modification	Yes
Type of modification	Correction of heterozygous 18bp mutation (c.1120del18)
Associated disease	Hereditary Hemorrhagic Telangiectasia type 2
Gene/locus	ACVRL1
Method of modification	CRISPR-Cas9
	Not applicable
Name of transgene or resistance	
Inducible/constitutive system	Not applicable
Date archived/stock date	18-03-2020
Cell line repository/bank	https://hpscreg.eu/cell-line/LUMCi030-A https://hpscreg.eu/cell-line/LUMCi030-A-1 https://hpscreg.eu/cell-line/LUMCi030-B https://hpscreg.eu/cell-line/LUMCi030-B-1
Ethical approval	Biopsies were taken with an informed consent at the St. Antonius Hospital, Nieuwegein, The Netherlands. The generation of the lines was approved by the Leiden University ethics committee under the P13.080 "Parapluprotocol: hiPSC"

1. Resource utility

Patients with a heterozygous c.1120del18 mutation in *ACVRL1* display Hereditary Hemorrhagic Telangiectasia (HHT) type 2, which is associated with regular bleedings, telangiectasia and arteriovenous malformations (Letteboer et al., 2005). iPSCs were generated from a patient carrying this mutation and CRISPR-Cas9 mediated repair was used to correct the mutation, hereby enabling in vitro disease modelling

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Table 1
Summary of lines.

iPSC line names	Abbreviation in Fig.s	Gender	Age	Ethnicity	Genotype of locus	Disease
LUMCi030-A	LUMC0110iALK04	Male	42	Caucasian	ACVRL1:c.1120del18	Hereditary Hemorrhagic Telangiectasia
LUMCi030-B	LUMC0110iALK10	Male	42	Caucasian	ACVRL1:c.1120del18	Hereditary Hemorrhagic Telangiectasia
LUMC030-A-1	iso03LUMC0110iALK04	Male	42	Caucasian	ACVRL1:no mutation	None (isogenic)
LUMC030-B-1	iso01LUMC0110iALK10	Male	42	Caucasian	ACVRL1:no mutation	None(isogenic)

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Transmission light microscopy	Normal	Fig. 1B
Phenotype	Pluripotency markers, qualitative analysis: Immunofluorescent staining	OCT3/4, SSEA4 & Nanog expressed	Fig. 1A
	Pluripotency markers, quantitative analysis: Flow cytometry	% of cells double positive for Nanog & SSEA4: LUMC0110iALK04: 85% iso03LUMC0110iALK04: 87% LUMC0110iALK10: 97% iso01LUMC0110iALK10: 96%	Fig. 1C
Genotype	Karyotype KaryoStat Resolution >1-2 Mb 2) G-banding Resolution 5-10 Mb	LUMC0110iALK04: 46XY iso03LUMC0110iALK04: 46XY LUMC0110iALK10: 46XY iso01LUMC0110iALK10: 46XY	1) Fig. 1G 2) Fig. 1H
Identity	STR analysis	22 loci tested, 100% matching identity	Available with the authors
Mutation analysis	TOPO TA cloning, Sanger sequencing	Heterozygous 18bp deletion	Fig. 1D
	Southern Blot OR WGS	Not performed	Not performed
Microbiology and virology	Mycoplasma	Mycoplasma tested by luminescence: Negative	Supplementary Table 1
Differentiation potential	Spontaneous differentiation or directed differentiation	LUMC0110iALK04, LUMC0110iALK10: areas with b3-tubulin+- (ectoderm), AFP+- (endoderm) and CD31+ (mesoderm) expressing cells iso03LUMC0110iALK04/iso01LUMC0110iALK10: Expression of PAX6, b3-tubulin (ectoderm), FOXA2, SOX17 (endoderm), Vimentin and NCAM (mesoderm)	Fig. 1F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	Not performed
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	Not performed
	HLA tissue typing	Not performed	Not performed

of HHT type 2 (Roman and Hinck, 2017).

2. Resource Details

Fibroblasts were isolated from a skin biopsy of a 42 year old male Hereditary Hemorrhagic Telangiectasia (HHT) type 2 patient with a heterozygous c.1120del18 mutation in *ACVRL1* and reprogrammed at passage three using oriP/EBNA1-based episomal vectors (Letteboer et al., 2005, Okita et al., 2011). The iPSC clones LUMC0110iALK04 and LUMC0110iALK10, displayed a typical morphology and a normal karyotype (passage 17 and 15 respectively) as assessed by the KaryoStat assay (Table 1Table 2, Fig 1B/G). Immunofluorescence staining showed homogenous expression of the pluripotency markers OCT3/4, NANOG and SSEA4 and flow cytometry analysis illustrated high percentages of pluripotency marker expression: 85% of NANOG$^+$/SSEA4$^+$ cells for LUMC0110iALK04 and 97% for LUMC0110iALK10 (Fig 1A/C, Table 2). Moreover, the iPSCs were able to differentiate into derivatives of all three germ layers upon spontaneous differentiation *in vitro* (Fig 1F). The heterozygous 18bp deletion in exon 8 of *ACVRL1* was confirmed by Sanger sequencing (Fig 1D) and the mutated allele was repaired by transfection with Cas9-ribonucleoprotein (RNP) complex, with a mutation-specific single guide RNA (sgRNA) and a single-stranded oligodeoxynucleotide (ssODN) containing the wild-type sequence as a donor template (Table 3). Single-cell derived colonies were screened for repair using the XCMI restriction enzyme, which only recognizes the wild-type sequence, and corrected clones were confirmed by Sanger sequencing (Fig 1E). The repaired iPSCs showed a typical morphology and a normal karyotype using KaryoStat at passage 6 after gene editing (Table 2, Fig 1B/G). As balanced translocations cannot be detected by KaryoStat, additionally G-banding was performed for the repaired iPSCs. Using this method, again both clones appeared to have a normal karyotype (Fig 1H). Moreover, the pluripotency markers OCT3/4, NANOG and SSEA4 were expressed at high levels (87% NANOG$^+$/SSEA4$^+$ cells for iso03LUMC0110iALK04 and 96% for iso01-LUMC0110iALK10) (Fig 1A/C) and the clones were able to differentiate into ectoderm, mesoderm and endoderm after directed differentiation (Fig 1F). All lines were mycoplasma negative (Supplementary Table 1). The origin of the isogenic pairs was confirmed by short tandem repeat (STR) analysis which fully matched the profile of the patient's somatic cells. Finally, the absence of off-target mutations was confirmed by Sanger sequencing of the top5-predicted sites by CRISPOR (crispor.tefor.net; data not shown) (Haeussler et al., 2016).

3. Materials and Methods

3.1. Reprogramming

A 4 mm skin biopsy was dissociated and fibroblasts were cultured in DMEM/F12 supplemented with 10% Fetal Bovine Serum, 1% Non-Essential Amino Acids, 1% Pen/Strep and 0.18% β-mercaptoethanol (all Gibco). At passage three, 5*10^5 cells were electroporated with 1 µg of episomal plasmids pCXLE-hOCT3/4, pCXLE-hSK and pCXLE-hUL (Addgene, #ID27076, #ID27078, #ID27080,) respectively, using the NHDF nucleofector kit with program U-023 of the NucleofectorII device

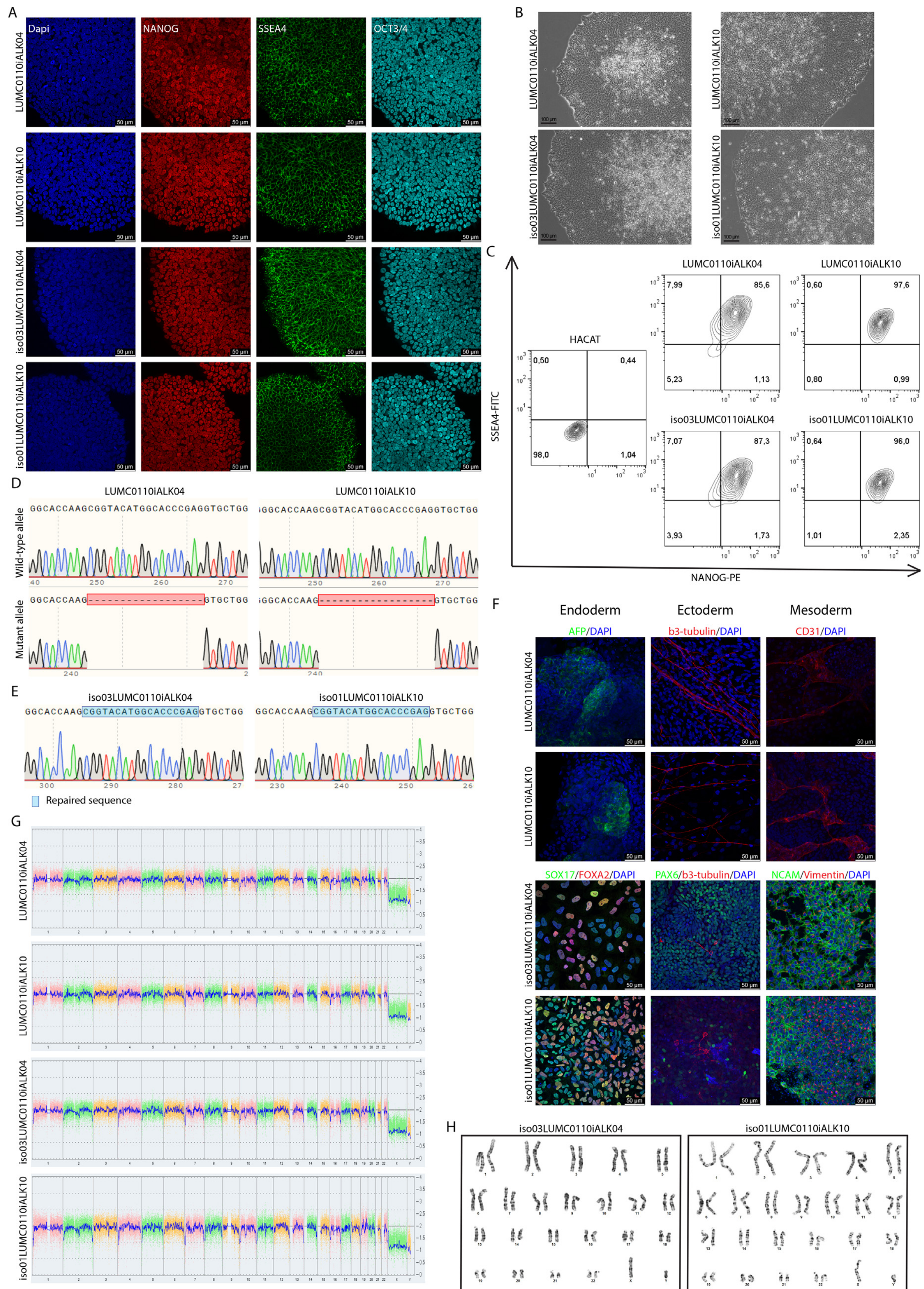

Fig. 1. Characterization of iPSC lines. A. Immunofluorescent staining for pluripotency markers NANOG, SSEA4 and OCT3/4. B. Morphology of the iPSC-colonies. C. Flow cytometry analysis of SSEA4 and NANOG. D. Sanger sequencing data of LUMC0110iALK04 and LUMC0110iALK10. E. Sanger sequencing data of iso03LUMC0110iALK04 and iso01LUMC0110iALK10. F. Immunofluorescent staining for endo-, ecto- and mesoderm markers after spontaneous (LUMC0110iALK04 and LUMC0110iALK10) or directed differentiation (iso03LUMC0110iALK04 and iso01LUMC0110iALK10). G. Results of KaryoStat assay. H. G-banding analysis.

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry	Antibody	Dilution	Company Cat # and RRID
Pluripotency Marker	mouse IgG2b anti-OCT3/4	1:100	Santa Cruz, Sc-5279, RRID: AB_628051
Pluripotency Marker	mouse IgG1 anti-NANOG	1:150	Santa Cruz, Sc-293121, RRID: AB_2665475
Pluripotency Marker	mouse IgG3 anti-SSEA4	1:30	Biolegend, #330402, RRID: AB_1089208
Pluripotency Marker	human IgG1 anti-SSEA4-FITC	1:25	Miltenyi, #130-098-371, RRID: AB_2653517
Pluripotency Marker	mouse IgG1 anti-NANOG-PE	1:5	BDBioscience, #560483, RRID: AB_1645522
Differentiation Marker, Ectoderm	mouse IgG2a anti-b3-tubulin	1:4000	Covance, #MMS-435P, RRID: AB_2313773
Differentiation Marker, Endoderm	Rabbit IgG anti-AFP	1:25	Quartett, #2011200530, RRID: AB_2716839
Differentiation Marker, Mesoderm	mouse IgG1 anti-PECAM-1	1:100	DAKO, #M0823, RRID: AB_2114471
Differentiation Marker, Ectoderm	Rabbit IgG anti-PAX6	1:200	Cell Signaling Technology, #60433, RRID: AB_2797599
Differentiation Marker, Endoderm	Goat IgG anti-SOX17	1:100	R&D, #AF1924, RRID: AB_355060
Differentiation Marker, Endoderm	Rabbit IgG anti-FOXA2	1:100	Millipore, #07-633, RRID: AB_390153
Differentiation Marker, Mesoderm	Rabbit anti-Vimentin	1:200	Cell Signaling Technology, #5741, RRID: AB_10695459
Differentiation Marker, Mesoderm	Mouse anti-NCAM	1:400	Cell Signaling Technology, #3576, RRID: AB_2149540
Secondary antibody	Alexa 647 Goat Anti-Mouse IgG2b	1:250	Invitrogen Cat# 21242, RRID: AB_2535811
Secondary antibody	Alexa 488 Goat Anti-Mouse IgG3	1:250	Invitrogen Cat# 21151, RRID: AB_2535784
Secondary antibody	Alexa 568 Goat Anti-Mouse IgG1	1:250	Invitrogen Cat# 21124, RRID: AB_2535766
Secondary antibody	Alexa 568 Goat Anti-Mouse IgG	1:500	Invitrogen Cat# A11031, RRID: AB_144696
Secondary antibody	Alexa 488 Donkey Anti-rabbit IgG	1:500	Invitrogen Cat# A21206, RRID: AB_2535792
Secondary antibody	Alexa 647 Donkey Anti-Goat IgG	1:250	Invitrogen Cat# A21447, RRID: AB_2535864
Primers			
	Target	**Forward/Reverse primer (5′-3′)**	
PCR mutation site	exon 8: *ACRVL1*	FW: TGCCTGCCTTGCCCACTCACATCT RV: AGGCAGATGGAGACGTGCACGGAGAT	
Sanger sequencing mutation site	exon 8: *ACRVL1*	FW: TGGGAACCTAGAGAGCACTACA RV: ACCCAAGATTTCACAGCCAGA	
Offtarget site 1, PCR & Sanger sequencing	intergenic: *RNU7-187P-NCAM1*	FW: AACAGCCACGGGCCTCCTTCATTTCTTT RV: AATCTGGGCCATAAGCCTGTCTGTCTCCTT	
Offtarget site 2 PCR & Sanger sequencing	intergenic: *RP11-953B20.2-RP11-953B20.1*	FW: CCCAACCCAGACCACCCTCGACGTATTT RV: TAGGCCACCCTGCATGCCCTGTTT	
Offtarget site 3 PCR & Sanger sequencing	intergenic: *MPP7-AC022021.1*	FW: GCAGAACTCCCTCATTGGTCGGCAAGAGAA RV: ACTCGCATCTGGCGAATGAACCTGCGTATC	
Offtarget site 4 PCR & Sanger sequencing	intergenic: *RP11-347D21.3-SNORD36*	FW: CTGGATTTAGCCTGAGGAGATG RV: GAAGGCAGAGGTGGCTATTT	
Offtarget site 5 PCR & Sanger sequencing	intergenic: *RP11-195B3.1-RP11-482E14.1*	FW: AGCTGACTGATGGCATTGTGCTTCCGTCTT RV: GCAGAGCCCTTCGGCTTACCCTGTATGTT	
crRNA			
	Target		**crRNA sequence -*PAM***
crRNAsense	c.1120del18 specific		CAACCCGAGAGTGGGCACCA -*AGG*
ssODN			
	Sequence		
ssODN for repair c.1120del18	CCTGGCTGTGATGCACTCACAGGGCAGCGATTACCTGGACATCGGCAACAACCCGAGA-GTGGGCACCAAGCGGTACATGGCACCCGAGGTGCTGGACGAGCAGATCCGCACGGAC-TGCTTTGAGTCCTACAAGTGGACTGACATCTGGGCCTTTGGCC		

(both Lonza) and subsequently cultured in ReproTeSR (Stem Cell Technologies) on Matrigel (Corning)-coated plates. iPSC colonies were picked manually and expanded in TeSR-E8 medium (Stem Cell Technologies) on VitronectinXF-coated plates (Stem Cell Technologies).

3.2. Cell culture

iPSCs were maintained at 37°C and 5% CO_2 in TeSR-E8 medium (Stem Cell Technologies) on VitronectinXF-coated plates (Stem Cell Technologies) with daily media changes. Once a week, iPSCs were passaged as small aggregates using Gentle Cell Dissociation Reagent (Stem Cell Technologies) according to manufacturer's protocol. Splitting ratio's ranged from 1:10 till 1:40 depending on cell confluency at the day of passaging.

3.3. Gene editing

LUMC0110iALK04 was cultured until passage 22 and LUMC0110iALK10 until passage 19 prior to gene editing. 1*10^5 cells were transfected with the Alt-R Cas9-RNP complex and the ssODN (both IDT) using the NEON Transfection System (Invitrogen) at 1200V/30ms/1pulse. Cells were plated in a Synthemax II-SC (Corning)-coated plate using TeSR-E8 with CloneR (Stem Cell Technologies). After recovery, 1000 cells were plated in a Synthemax II-SC-coated 10cm dish in TeSR-E8 with CloneR. Half of a single cell-derived colony was used for DNA isolation with QuickExtract solution (Lucigen). The region of

interest was PCR-amplified using Terra PCR Direct Polymerase Mix (TaKaRa) and a Bio-Rad S1000 Thermal Cycler with the following parameters: 1: 2min at 98°C, 2: 10sec at 98°C, 3: 1min/kb at 68°C, 35 cycles. Positive clones were identified by successful cutting of the PCR amplified *ACVRL1* fragment by restriction enzyme XCMI according to manufacturer's instructions.(New England Biolabs). The repaired allele in the corrected clones was confirmed by Sanger sequencing, performed by the Leiden Genome Technology Centre (LGTC) using the ABI3730xl system.

3.4. Flow cytometry

Single cells were prepared with Gentle Cell Dissociation Reagent (Stem Cell Technologies) and fixed and permeabilized using the FIX & PERM Cell Fixation & Permeabilization Kit (Invitrogen) according to manufacturer's protocol. Cells were stained with the directly conjugated antibodies against SSEA4 and NANOG and subsequently measured using the MACSQuant VYB (Miltenyi Biotech). Data was analyzed with FlowJo version 10.6.1.

3.5. Trilineage differentiation

iPSCs were either subjected to spontaneous differentiation (LUMC0110iALK04 & LUMC0110iALK10) in DMEM/F12 + 20% FBS (Gibco) for 21 days or directed differentiation (iso03LUMC0110iALK04, iso01LUMC0110iALK10) using STEMdiff™ Trilineage ectoderm-, endoderm- and mesoderm media according to manufacturer's protocol (Stem Cell Technologies).

3.6. Phase contrast imaging and immunofluorescent (IF) staining

Phase contrast pictures were taken of live cells 6 days after passage using a Nikon eclipse T1 microscope with a 10x objective. For IF staining, cells were fixed with 2% PFA for 30 min at RT. Subsequently, cells were blocked and permeabilized using 0.1% TritonX-100 + 4% normal swine serum (Jackson) in PBS for 1h at RT. Primary antibodies were diluted in PBS with 4% normal swine serum (Jackson) and applied O/N (4°C), followed by the secondary antibodies in PBS for 1 hour (RT) (Table 3). DNA counterstaining was performed with DAPI (1:1000, Invitrogen). Images were taken with a 40x objective using a Leica TCS SP8 confocal microscope.

3.7. Evaluation of off-target effects

Top5 off-target sites were predicted using the CRISPOR website (crispor.tefor.net) (Haeussler et al., 2016). PCR products ranging from 230 up to 908bp covering the predicted sites were screened by Sanger sequencing, performed by the Leiden Genome Technology Centre (LGTC) using the ABI3730xl system.

3.8. Mycoplasma detection

The mycoplasma status was assessed using the MycoAlert™ mycoplasma detection kit (Lonza, #LT07-418) following the manufacturers protocol.

3.9. Short Tandem Repeat (STR) analysis

STR-analysis was performed by the LUMC Forensic Laboratory for DNA-research (FLDO), using the PowerPlex Fusion System 5C autosomal STR kit (Promega) as previously described (Westen et al., 2014).

3.10. Karyotyping

G-banding analysis was conducted at the Laboratory of Clinical Genetics Leiden (LDGA), according to standard procedures. A total of 20 metaphases was analyzed for each line, for iso03LUMC0110iALK04 at passage 5 and for iso01LUMC0110iALK10 at passage 7 after gene editing. The KaryoStat assay was performed for LUMC0110iALK04 at passage 17, LUMC0110iALK10 at passage 15 and for iso03LUMC0110iALK04 and iso01LUMC0110iALK10 at passage 6 after gene editing according to manufacturer's instructions (ThermoFisher Scientific).

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2020.101786.

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