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The branching of life: human iPSC-based angiogenesis-on-chip

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Chapter 4

Generation of patient-derived and gene-corrected hiPSC lines from Hereditary Hemorrhagic Telangiectasia type 2 patients with ACVRL1 c.1042delG mutation

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Abstract

Hereditary Hemorrhagic Telangiectasia type 2 (HHT2) is a vascular disorder caused by mutations in *ACVRL1*. We generated human induced pluripotent stem cell (hiPSC) lines from two HHT2 patients with a heterozygous 1 bp deletion in exon 7 of *ACVRL1* (c.1042delG) by reprogramming skin fibroblasts. Gene-corrected isogenic hiPSCs were created using CRISPR-Cas9. All lines displayed normal karyotypes, expressed markers of the undifferentiated state, differentiated into all germ layers *in vitro*, and showed no off-target effects. These patient-derived, genetically matched hiPSC pairs provide a robust platform for modeling HHT2 *in vitro* and investigating molecular mechanisms of pathogenesis.

Resource Table:

Unique stem cell lines identifier	1. LUMCi017-A 2. LUMCi018-A 3. LUMCi017-A-1 4. LUMCi017-A-2 5. LUMCi018-A-1 6. LUMCi018-A-2
Alternative name(s) of stem cell lines	1. LUMC0111iALK, LUMC0111iALK07 2. LUMC0112iALK, LUMC0112iALK08 3. Iso01LUMC0111iALK07 4. Iso02LUMC0111iALK07 5. Iso01LUMC0112iALK08 6. Iso02LUMC0112iALK08
Institution	Leiden University Medical Center, LUMC, The Netherlands
Contact information of distributor	Valeria V. Orlova (v.orlova@lumc.nl)
Type of cell lines	hiPSC
Origin	Human
Additional origin info required	Age: 52 (LUMCi017-A) and 55 (LUMCi018-A) Sex: Male (LUMCi017-A) and Male (LUMCi018-A) Ethnicity: Caucasian (LUMCi017-A) and Caucasian (LUMCi018-A)
Cell Source	Skin fibroblasts
Clonality	Clonal
Method of reprogramming	Episomal vectors: *OCT3/4*, *SOX2*, *KLF4*, *LMYC* and *LIN28*
Genetic Modification	Yes
Type of Genetic Modification	Inherited and CRISPR-Cas9-mediated correction of *ACVRL1* c.1042delG mutations
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	Detection of the Epstein-Barr virus nuclear antigen 1 (*EBNA1*) by qPCR
Associated disease	Hereditary Hemorrhagic Telangiectasia type 2 (HHT2)
Gene/locus	*ACVRL1* (chr.12q13.13)
Date archived/stock date	27-01-2025
Cell line repository/bank	https://hpscreg.eu/cell-line/LUMCi017-A https://hpscreg.eu/cell-line/LUMCi018-A https://hpscreg.eu/cell-line/LUMCi017-A-1 https://hpscreg.eu/cell-line/LUMCi017-A-2 https://hpscreg.eu/cell-line/LUMCi018-A-1 https://hpscreg.eu/cell-line/LUMCi018-A-2
Ethical approval	Leiden University Medical Center ethics committee - P13.080 (Parapluprotocol: hiPSC).

Resource utility

Mutations in *ACVRL1* are a common cause of vascular dysplasia in patients diagnosed with Hereditary Hemorrhagic Telangiectasia type 2 (HHT2) (Snodgrass et al., 2021), with the c.1042delG variant reported in the Dutch population (Letteboer et al., 2005). Human iPSC-derived models offer a valuable alternative to the limited availability of patient-specific material for studying HHT2 pathophysiology *in vitro* (Bouma et al., 2020; Orlova et al., 2022).

Resource Details

HHT2 is characterized by small, dilated blood vessels, termed telangiectasias, in mucocutaneous regions that lead to severe, recurrent nosebleeds. Larger arteriovenous malformations (AVMs) can develop in internal organs such as the brain, lungs, liver, and intestines, where they are potentially life-threatening if left untreated (Hermann et al., 2025). HHT is classified as rare disease with a prevalence of 1:5000 globally (Shovlin, 2010). The pathomolecular mechanisms driving the disease remain incompletely understood, partially due to the lack of *in vitro* models that closely resemble the *in vivo* situation. In this report, we generated multiple patient-derived, genetically matched hiPSC pairs to better model HHT type 2 *in vitro*.

Skin biopsies were collected from two male HHT2 patients (aged 52 and 55) carrying a heterozygous c.1042delG mutation in exon 7 of *ACVRL1*. Isolated fibroblasts were reprogrammed at passage three using oriP/EBNA-1-based episomal vectors. The presence of the *ACVRL1* c.1042delG mutation in patient-derived hiPSCs was confirmed by targeted Sanger sequencing (Figure 1A). Next, we employed CRISPR-Cas9 to genetically correct the genetic variant using a mutation-specific single guide RNA (sgRNA) and single-stranded donor template (ssODN; Table 2). The ssODN contained two silent mutations to prevent recutting by Cas9 and to facilitate screening of successfully edited cells. Corrected clones were identified via AlwI restriction enzyme digestion of genomic DNA and validated by targeted Sanger sequencing (Figure 1B). Examination of the top three predicted off-target sites revealed no alternations (Data not shown; crispor.tefor.net)(Haeussler et al., 2016). Parental and gene-corrected hiPSC lines

homogenously expressed markers of the undifferentiated state NANOG, SSEA4, OCT3/4, and TRA-1-60 as assessed by immunocytochemistry (Figure 1C) and fluorescence-activated cell sorting (Figure 1D). Furthermore, all hiPSC lines displayed the typical cellular morphology (Supplementary Figure 1A), and were able to differentiate in vitro towards endoderm (AFP, SOX17, FOXA2), ectoderm (β3-tubulin, PAX6) and mesoderm (CD31, NCAM, Brachyury) lineages (Figure 1E and 1F). KaryoStat analysis revealed a normal karyotype for each line (Supplementary Figure 1B), whereas short tandem repeat (STR) analysis of the lines verified the genetic identity between isogenic pairs (Table 1). All lines tested negative for mycoplasma (Suppl. Table 1) and absence of the episomal reprograming vectors (Suppl. Table 2).

Materials and Methods

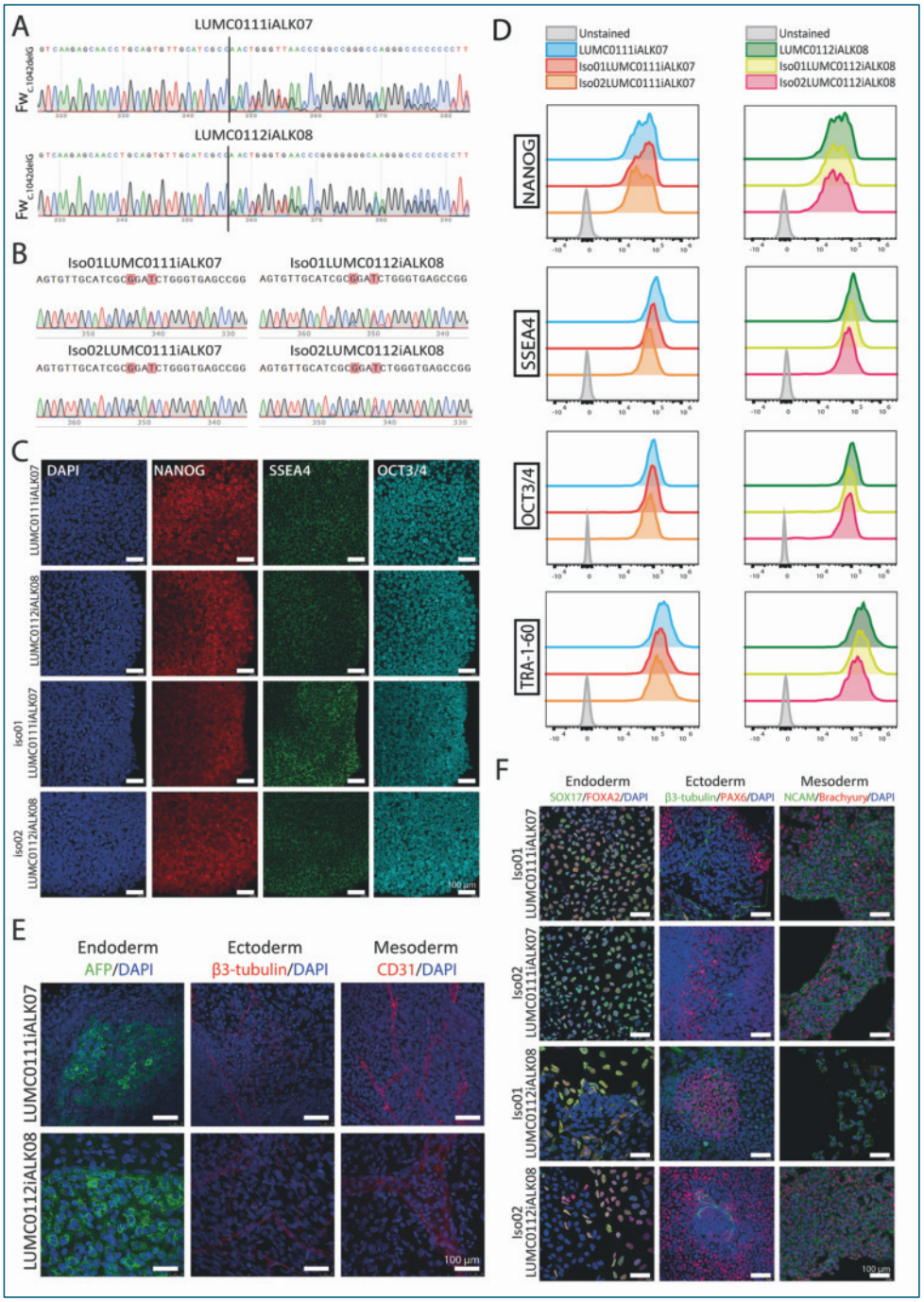
Reprogramming and hiPSC maintenance

Fibroblasts from dissociated skin biopsies were cultured in DMEM/F12 with 10% FBS, non-essential amino acids, β-mercaptoethanol, and penicillin/streptomycin (Gibco). At passage three, 5×10^5 cells were electroporated with 1 µg each of pCXLE-hOCT3/4, pCXLE-hSK, and pCXLE-hUL (Addgene #27076, #27078, #27080) using the NHDF Nucleofector Kit and Nucleofector II (program U-023, Lonza). Cells were cultured in ReproTeSR (Stem Cell Technologies) on coated plates with Matrigel (Corning). hiPSC colonies were manually picked and expanded in TeSR-E8 on VitronectinXF (Stem Cell Technologies)-coated plates. Media was changed daily, and cells were passaged weekly (1:40) using Gentle Cell Dissociation Reagent and maintained at 37°C, 5% CO_2.

Gene editing

HiPSCs were cultured beyond passage 10 before transfection with Alt-R® Cas9-RNP complex and ssODN (IDT) using the NEON Transfection System (1×10^5 cells, 1200 V, 30 ms, 1 pulse; Invitrogen). Transfected cells were plated on Synthemax II-SC-coated plates in TeSR-E8 with CloneR™. After seven days, single-cell colonies were seeded (1000 cells/10 cm dish) in TeSR-E8 with CloneR™. Genomic DNA from each clone was extracted using QuickExtract (Lucigen). Initial screening used AlwI digestion (NEB), and positive clones were validated by Sanger sequencing (ABI3730xl, LGTC).

Figure 1. Characterization of hiPSC lines.



Imaging and immunocytochemistry

Phase-contrast images were captured using an Eclipse T1 microscope (Nikon) with a 10× objective. For immunocytochemistry, cells were fixed in 2% PFA/PBS for 30 min at room temperature, then blocked/permeabilized with 0.1% Triton X-100 and 4% normal swine serum (Jackson) in PBS for one hour. Primary antibodies were incubated overnight at 4 °C, followed by secondary staining for one hour at room temperature. Nuclei were counterstained with DAPI (1:1000, Invitrogen), and images acquired using a Leica TCS SP8 confocal microscope.

Flow cytometry

Single-cell suspensions were generated using Gentle Cell Dissociation Reagent for five minutes at room temperature, followed by gentle pipetting. Fixation and permeabilization were performed using the FIX & PERM Kit (Invitrogen) according to the manufacturer's instructions. Cells were stained at passage 15 for pluripotency markers (Table 2) and analyzed using the Cytek Northern Lights and SpectroFlo software (Cytek Biosciences).

Germ layer differentiation

hiPSCs underwent either spontaneous (LUMC0111iALK07 and LUMC0112iALK08) or directed differentiation (iso01LUMC0111iALK07, iso02LUMC0111iALK07, iso01LUMC0112iALK08, and iso02LUMC0112iALK08) in monolayers. For spontaneous differentiation, cells were maintained in DMEM/F12 with 20% FBS (Gibco) for 21 days. Directed differentiation was performed using STEMdiff Trilineage media for endoderm, ectoderm, and mesoderm per manufacturer's instructions (Stem Cell Technologies).

Karyotyping

Genomic integrity was assessed using the KaryoStat™ assay according to the manufacturer's instructions (ThermoFisher Scientific).

Short Tandem Repeat (STR) analysis

STR profiling was performed by the LUMC Forensic Laboratory for DNA Research using the PowerPlex Fusion System 5C autosomal STR kit (Promega) following established protocols (Westen et al., 2014).

Mycoplasma detection

The absence of Mycoplasma was evaluated using the MycoAlert™ Mycoplasma Detection Kit (Lonza, #LT07-418) following the manufacturer's instructions (Supplementary Table 1).

Declaration of competing interest

The authors declare no financial interests or personal relationships that may have impacted the outcome of this work.

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Table 1: Characterization and validation.

Classification	Test	Result	Data
Morphology	Brightfield microscopy	Normal morphology	Supplementary Fig. 1 panel A
Phenotype	Qualitative analysis by IF of the pluripotency markers	All lines are positive for OCT4, SSEA4 and NANOG.	Fig. 1 panel C
	Quantitative analysis of the pluripotency markers by FACS	Percentage of cells positive for NANOG, SSEA4, OCT3/4 and TRA-1-60: LUMC0111iALK07: 99%, 100%, 100%, 100% LUMC0112iALK08: 99.9%, 100%, 100%, 100% Iso01LUMC0111iALK07: 99,9%, 100%, 100%, 100% Iso02LUMC0111iALK07: 100%, 99.8%, 100%, 100% Iso01LUMC0112iALK08: 100%, 99.8%, 100%, 100% Iso02LUMC0112iALK08: 100%, 99.8%, 100%, 99.7%	Fig. 1 panel D
Genotype	Karyotype: KaryoStat, Resolution >1-2Mb	No chromosomal abnormalities for all hiPSC clones	Supplementary Fig. 1 panel B
Identity	STR analysis	23 loci tested; all sites matched.	Submitted in archive with journal
Mutation analysis	TOPO TA cloning, Sanger sequencing	Heterozygous mutation: *ACVRL1* c.1042delG	Fig. 1 panel A and B
	Southern Blot OR WGS	N/A	N/A
Off-target nuclease activity	Top 3 predicted potential off-target sites by PCR	No mutations detected in predicted sites	Data not shown
Microbiology and virology	Mycoplasma	Mycoplasma tested by luminescence: Negative	Supplementary material, Table 1
Reprogramming transgene loss	Quantitative analysis of the EBNA1 gene by PCR	EBNA1 not detected in LUMC0111iALK07 and LUMC0112iALK08	Supplementary material, Table 2
Differentiation potential	Spontaneous differentiation and directed differentiation	Expression of the three germ layer makers: endoderm (AFP, SOX17,FOX2A), ectoderm (β3-Tubulin, PAX6), and mesoderm (CD31, NCAM, Brachyury)	Fig. 1 panel E and F

Table 2: Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry				
	Antibody	Dilution	Company Cat #	RRID
Pluripotency Marker (FACS)	Human IgG1 anti-SSEA4-Vioblue	1:50	Miltenyi #130-128-254	AB_2921840
Pluripotency Marker (FACS)	Human IgG1 Anti-Nanog-60-vioB515	1:50	Miltenyi #130-129-260	AB_2905342
Pluripotency Marker (FACS)	Human IgG1 Anti-Oct3/4-60-APC	1:50	Miltenyi #130-117-821	AB_2784445
Pluripotency Marker (FACS)	Human IgG1 Anti-TRA-1-60-PE	1:50	Miltenyi #130-122-965	AB_2801990
Pluripotency Marker (IF)	Mouse IgG2b anti-OCT3/4	1:100	Santa Cruz Sc-5279	AB_628051
Pluripotency Marker (IF)	Mouse IgG1 anti-NANOG	1:150	Santa Cruz Sc-293121	AB_2665475
Pluripotency Marker (IF)	Mouse IgG3 anti-SSEA4	1:30	Biolegend #330402	AB_1089208
Differentiation Marker, Ectoderm	Mouse IgG2a anti-b3-tubulin	1:4000	Covance #MMS-435P	AB_2313773
Differentiation Marker, Ectoderm	Rabbit IgG anti-PAX6	1:200	Cell Signaling Technology #60433	AB_2797599
Differentiation Marker, Endoderm	Rabbit anti-AFP	1:25	Quartett #2011200530	AB_2716839
Differentiation Marker, Endoderm	Goat IgG anti-SOX17	1:100	R&D #AF1924	AB_355060
Differentiation Marker, Endoderm	Rabbit IgG anti-FOXA2	1:100	Millipore #07-633	AB_390153
Differentiation Marker, Mesoderm	Mouse anti-PECAM-1	1:100	DAKO #M0823	AB_2114471
Differentiation Marker, Mesoderm	Alexa 488 Rabbit Anti-Brachyury (D2Z3J)	1:200	Cell Signaling Technology #94663S	AB_2799983
Differentiation Marker, Mesoderm	Mouse anti-NCAM	1:400	Cell Signaling Technology #3576	B_2149540
Secondary antibody	Alexa 647 Goat anti-Mouse IgG2b	1:250	Invitrogen Cat# A-21242	AB_253581
Secondary antibody	Alexa 488 Goat anti-Mouse IgG3	1:250	Invitrogen Cat# A-21151	AB_2535784
Secondary antibody	Alexa 568 Goat anti-Mouse IgG1	1:250	Invitrogen Cat# A-21124	AB_2535766
Secondary antibody	Alexa 568 Goat anti-Mouse IgG (H+L)	1:500	Invitrogen Cat# A-11031	AB_144696
Secondary antibody	Alexa 488 Donkey anti-Rabbit IgG (H+L)	1:500	Invitrogen Cat# A-21206	AB_2535792
Secondary antibody	Alexa 647 Donkey anti-Goat IgG (H+L)	1:250	Invitrogen Cat# A-21447	AB_2535864
Primers and Oligonucleotides used				
	Target	**Size of band**	**Forward/Reverse primer (5′-3′)**	
PCR mutation site	Exon 7: ACRVL1	*912*	FW: GCAAGCCACTAAGCCAGGCCCAGAC RV:AGGCAGATGGAGACGTGCACGGAGAT	
Sanger sequencing mutation site	Exon 7: ACRVL1	-	FW: CTTAGCAGTGACCCAGTCCATTC RV: CCCAGGTACTCTTTGCTTGTGTAG	

Off target site 1, PCR & Sanger sequencing	Intergenic: HES3-GPR153	*738*	FW: ACTTCGGTGGTGGGAGGCTCTCAAGT RV: ACCTGGCATTCCAAGGCTGGTTTCGTAGA
Off target site 2 PCR & Sanger sequencing	Exon: SLC6A12	*349*	FW: AGCTCAACAGAAACGGAGCAAGGCAAAGG RV: AGCCTGATGGGACAATACCCTGTTGGAAGT
Off target site 3 PCR & Sanger sequencing	Intronic: KHDRBS3	*230*	FW: TGGGAAAGAGGAAAGCCTGAGAGGGTACAT RV: ACCACAACAATTGGCCACGCAACACA
Reprogramming transgene loss, qPCR.	HK2P	*20*	FW: GCCGACTCTTGTATTGCCTG Reverse: TATTGTAGCACGGCCGGAAA
Reprogramming transgene loss, qPCR.	EBNA1	*20*	FW: CAAGGAGGTTCCAACCCGAA Reverse: GACCCAAGTTCCTTCGTCGG

sgRNA	**Target**	**sgRNA sequence - PAM**
sgRNA sense	*ACVRL1* c.1042delG specific	GCAGTGTTGCATCGCCACCT - **GGG**

ssODN	**Sequence (nucleotide-of-interest in red and silent mutations underlined)**
Repair template	GGAGATCTTCGGTACACAGGGCAAACCAGCCATTGCCCACCGCGACTTCAAGAGCCGCAATGTGCTGGTCAAGAGCAACCT GCAGTGTTGCATCGC**G**GA**T**CTGGGTGAGCCGGGCGGGGCAGGGGCGCGCCCTTCACAGGTGGGCGGAGCTTGTGCGCTCTC CTCTCCTTTGCCTGTGGGCGGTGACCATGATTAGC

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	Measurement A	**Measurement B**	**Ratio – measurement B/A**	**Mycoplasma**
Positive control	210	2520	12	Positive
Negative control	202	98	0.485	Negative
LUMC0111iALK07	268	178	0.664	Negative
Iso01LUMC0111iALK07	484	220	0.454	Negative
Iso02LUMC0111iALK07	545	252	0.463	Negative
LUMC0112iALK08	272	172	0.632	Negative
Iso01LUMC0112iALK08	188	106	0.563	Negative
Iso02LUMC0112iALK08	168	96	0.571	Negative

Supplementary Table 1: Mycoplasma test

Interpretation ratio B/A
Negative <0.9/1.0
Positive >1.2

Suppl. Figure 1: Morphological characterization and KaryoStat of hiPSC lines.