Modeling vascular disease using self-assembling human induced pluripotent stem cell derivatives in 3D vessels-on-chip

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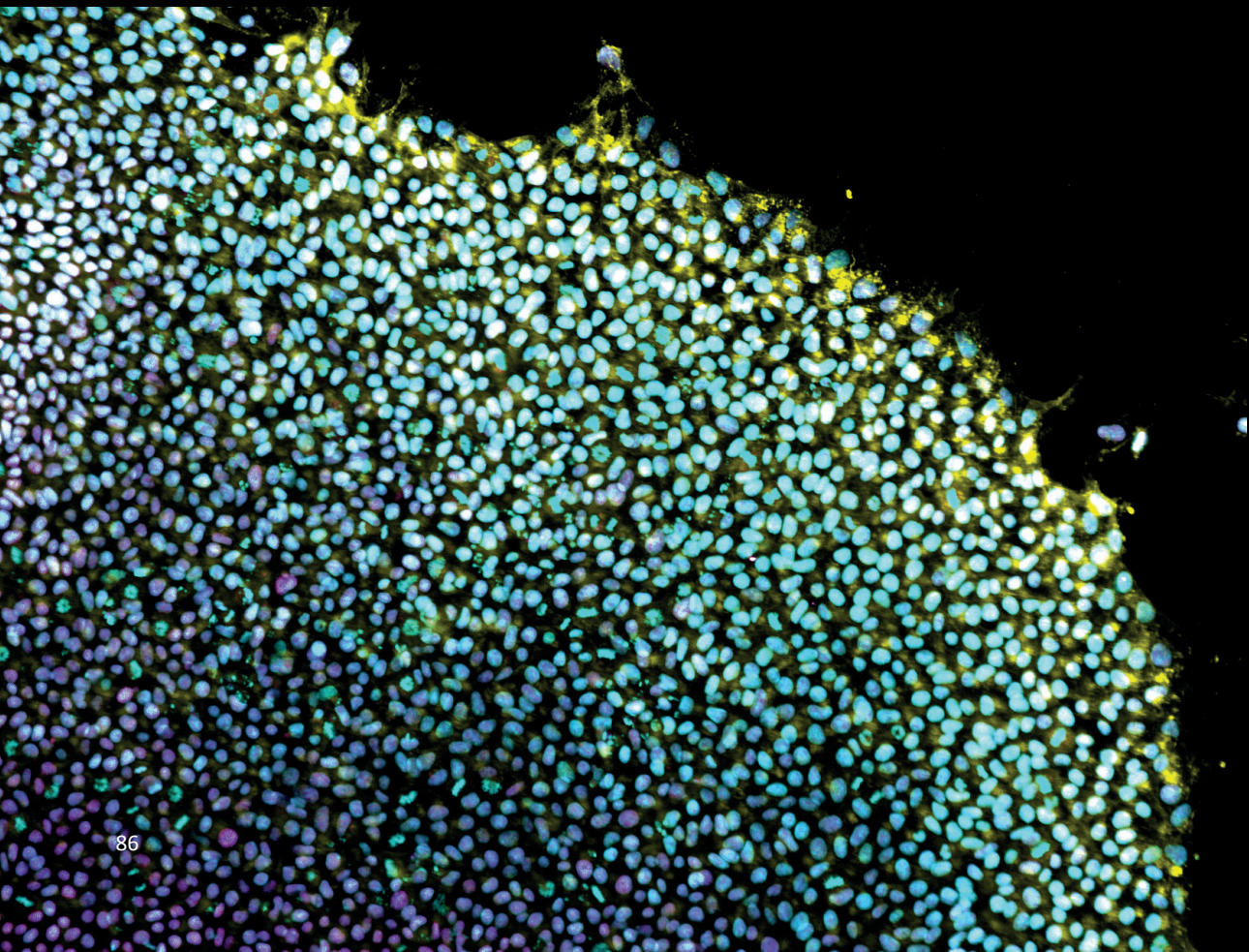

Chapter 3

Genetic repair of a human induced pluripotent cell line from patient with Dutch-type cerebral amyloid angiopathy

Abstract

Dutch-type cerebral amyloid angiopathy (D-CAA), also known as hereditary cerebral haemorrhage with amyloidosis-Dutch type (HCHWA-D), is an autosomal dominant disorder caused by a G to C transversion in codon 693 of the amyloid precursor protein (APP) that results in a Gln-to-Glu amino acid substitution. CRISPR-Cas9 editing was used for genetic correction of the mutation in a human induced pluripotent stem cell (hiPSC-) line established previously. The isogenic hiPSCs generated showed typical pluripotent stem cell morphology, expressed all markers of undifferentiated state, displayed a normal karyotype and had the capacity to differentiate into the three germ layers.

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Resource Table

Unique stem cell lines identifier	LUMCi005-A-3 (https://hpscreg.eu/cell-line/LUMCi005-A-3)
	LUMCi005-A-4 (https://hpscreg.eu/cell-line/LUMCi005-A-4)
Alternative names of stem cell lines	Iso1LUMC0074iHCHWAD01
	Iso3LUMC0074iHCHWAD01
Institution	Leiden University Medical Center, LUMC
Contact information of distributor	Valeria V. Orlova (v.orlova@lumc.nl)
	Dennis M. Nahon (d.nahon@lumc.nl)
	Christian Freund (c.m.a.h.freund@lumc.nl)
Type of cell lines	hiPSCs
Origin	Human
Additional origin info	Age: 56
	Sex: Female
	Ethnicity: Caucasian
Cell Source	Fibroblasts
Method of reprogramming	Sendai virus, OCT3/4, SOX2, KLF4 and MYC
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	qRT-PCR
Cell culture system used	hiPSCs cultured on Vitronectin in mTeSR plus
Type of Genetic Modification	Genetic correction of heterozygous point mutation in the APP gene
Associated disease	Dutch-type cerebral amyloid angiopathy (D-CAA), also known as hereditary cerebral haemorrhage with amyloidosis-Dutch type (HCHWA-D).
Gene/locus	APP 21q21.3
	Corrected Heterozygous APP c.2077 C >G; c.2082 T >C; c.2085 G >C
Method of Modification	CRISPR/Cas9
User-customisable nuclease (UCN) delivery method	Electroporation

All double-stranded DNA genetic material molecules introduced into the cells	N/A
Analysis of the nuclease-targeted allele status	Sequencing of the targeted allele
Method of the off-target nuclease activity prediction and surveillance	Targeted PCR/sequencing
Descriptive name of the transgene	N/A
Eukaryotic selective agent resistance cassettes (including inducible, gene/cell type-specific)	N/A
Inducible/constitutive expression system details	N/A
Date archived/stock date	27/02/2020
Cell line repository/bank	https://hpscreg.eu/cell-line/LUMCi005-A-3 https://hpscreg.eu/cell-line/LUMCi005-A-4
Ethical approval	NL45478.058.13/P13.080, Medical Ethical Committee(MEC), Leiden University Medical Center (LUMC).
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	N/A

Table 1: Characterization and validation

Classification	Test	Result	Data
Morphology	Brightfield microscopy	Normal morphology	Fig. 1 panel A
Phenotype	Qualitative analysis of IF staining	Positive immunostaining markers of undifferentiated state: SSEA4, OCT3/4 & NANOG	Fig. 1 panel D
	Quantitative analysis by FACS	Increased fluorescent signal over negative control of markers of undifferentiated state: SSEA4, OCT3/4 & NANOG	Fig. 1 panel B

Karyotype	G-band Karyotyping 5-10Mb	Normal karyotype: 46, XX for both lines	Fig. 1 panel E
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site and sequencing	PCR + Sanger sequencing confirmed presence edited allele (APP c.2077 C >G; c.2082 T >C; c.2085 G >C)	Fig. 1 panel C
	Evaluation of the homozygous status of introduced genomic alteration(s)	PCR + Sanger sequencing confirmed presence of edited allele (APP c.2077 C >G; c.2082 T >C; c.2085 G >C)	Fig. 1 panel C
	Transgene-specific PCR	N/A	N/A
Verification of the absence of random plasmid integration events	PCR/Southern	N/A	N/A
Parental and modified cell line genetic identity evidence	Microsatellite PCR (mPCR) OR	Not performed	N/A
	STR analysis	24 sites tested; all sites matched	Submitted in archive with journal
Off-target nuclease activity analysis	PCR across top 5 predicted top likely off-target sites	Demonstration of the lack mutations in predicted off-target sites	Supplementary Fig. S1
Specific pathogen-free status	Mycoplasma	RT-PCR analysis: Negative	Supplementary Table S1
Multilineage differentiation potential	Short-term differentiation in vitro by IF analysis	Positive immunostaining of the three germ layer markers: ectodermal (NES, PAX6), endodermal (FOXA2, GATA4), mesodermal (T, VIM)	Fig. 1F
Donor screening (OPTIONAL)	N/A	N/A	N/A

Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Resource utility

D-CAA is a neurodegenerative disorder, caused by a heterozygous mutation in exon 17 of the APP gene on chromosome 21 (E22Q; c.2077 G > C)[1]. The mutation results in accumulation of amyloid beta (Aβ) inside the cerebral vascular wall. D-CAA patient hiPSC lines and CRISPR-Cas9 corrected isogenically paired hiPSC-lines are a valuable tool for studying the underlying disease mechanisms.

Resource Details

D-CAA is caused by a point mutation in the APP gene resulting in severe amyloid deposition in the cerebral vascular wall, vascular dementia and recurrent hemorrhagic strokes from the fifth decade of life onwards, significantly reducing life expectancy[2]. The heterozygous mutation in the APP gene, results in a Gln-to-Glu amino acid substitution leading to charge alterations in the Aß protein[3]. The altered Aß is less efficiently degraded and cleared over the blood-brain barrier and induces death of cerebrovascular smooth muscle cells, reducing the stability of the vascular wall. Previously generated D-CAA patient hiPSC-lines[4] have been useful for modeling certain aspects of the disease[5]. In the current study, isogenic lines were created to further this research by correcting the mutation in the hiPSC-line generated previously using a Cas9-ribonucleoprotein (RNP) complex, with a mutation-specific single guide RNA (sgRNA) and a single-stranded oligodeoxynucleotide (ssODN) donor template containing the wildtype sequence with two additional silent mutations, one to introduce a AatII restriction site and the other to disrupt the protospacer adjacent motif (PAM) site (Table 2). Two different guide RNAs (gRNAs) were used in separate targeting experiments of the same line, which resulted in an isogenically corrected line with two clones generated using the first sgRNA and the two other clones generated using the other sgRNA. Single-cell derived colonies were screened for repair using the AatII restriction of PCR amplified APP exon 17 region. One clone per sgRNA, hereafter named LUMCi005-A-3 (gRNA1), LUMCi005-A-4 (gRNA2), was selected and characterized (Table 1). Correction of the mutation (c.2077 C >G) and incorporation of silent mutations was confirmed by Sanger sequencing (Fig 1C). The isogenic clones displayed typical stem cell morphology with high nucleus to cytoplasm ratio (Fig. 1A) and a normal karyotype was confirmed by G-banding (Fig. 1E). The pluripotency status was confirmed by expression and localization of markers of the undifferentiated state: SSEA4, NANOG and OCT3/4, using flow-cytometry (Fig 1B) and Immunofluorescence staining (Fig 1D). The differentiation potential of the lines was confirmed by short-term differentiation assay *in vitro*, with subsequent immunofluorescence staining for markers of the three germ layers; NESTIN and PAX6 (ectoderm), FOXA2 and GATA4 (endoderm), Brachyury (T), CDX2 and Vimentin (mesoderm) (Fig 1F). Clones were tested negative for

mycoplasma (Supplemental Table 1). Finally, the absence of off-target mutations was confirmed by Sanger sequencing of the five most likely off-target sites within coding regions as predicted by CRISPOR (crispor.tefor.net; Fig S1)[6].

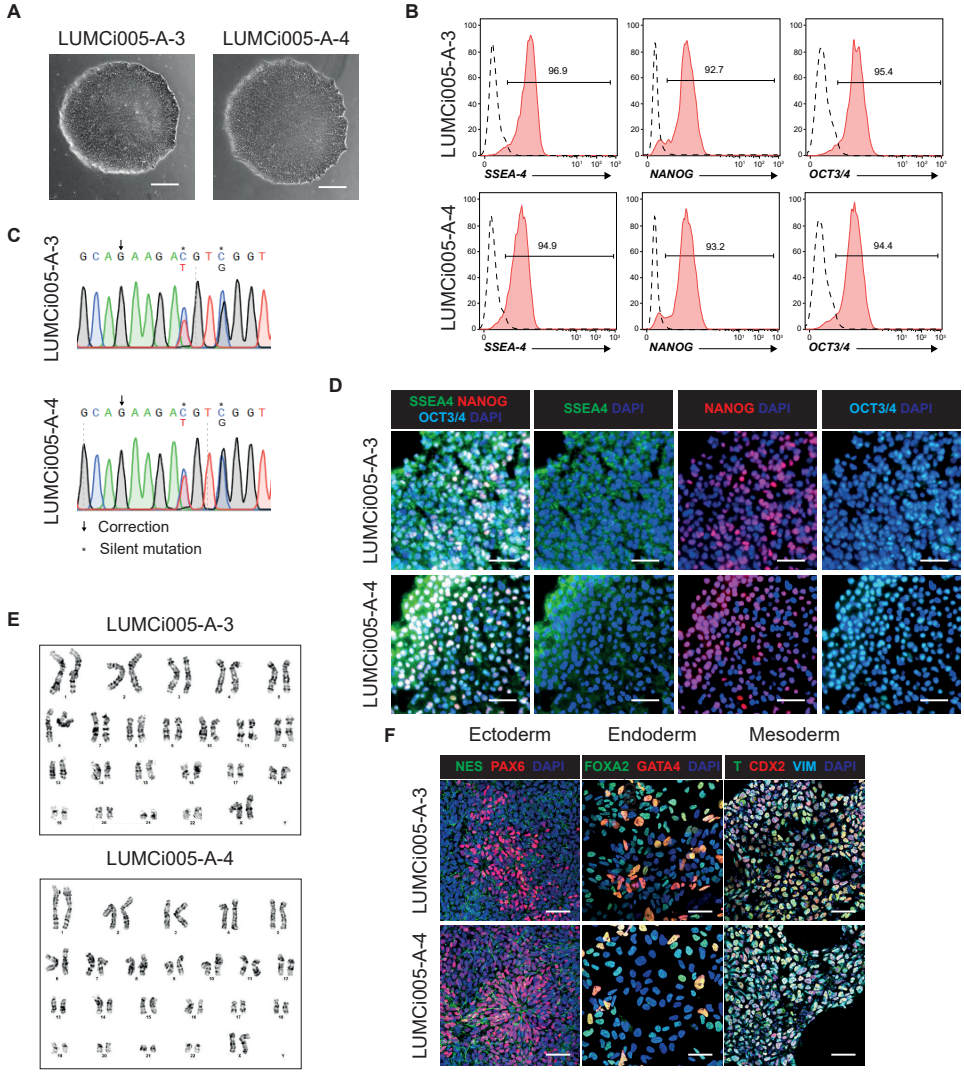

Figure 1: Generation and characterization of hiPSC lines LUMCi005-A-3, LUMCi005-A-4. Lines are CRISPR-Cas9 edited to correct an autosomal mutation in the amyloid precursor protein (APP) in a Dutch-type cerebral amyloid angiopathy (D-CAA) patient line. *Scale bar in A: 100 µm, scale bars in D and F: 50 µm.*

Table 2: Reagents details

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Markers of the undifferentiated state (FACS)	BV421 Mouse Anti-OCT3/4	1:25 (100k cells in 80 ul)	BD Biosciences Cat# 565644, RRID:AB_2739320
Markers of the undifferentiated state (FACS)	Mouse Anti-Human NANOG Monoclonal Antibody, PE Conjugated	1:5 (100k cells in 80 ul)	BD Biosciences Cat# 560483, RRID:AB_1645522
Markers of the undifferentiated state (FACS)	Anti-SSEA-4-FITC antibody	1:25 (100k cells in 80 ul)	Miltenyi Biotec Cat# 130-098-371, RRID:AB_2653517
Markers of the undifferentiated state (Immunocytochemistry)	Mouse IgG2b anti-OCT3/4	1:100	Santa Cruz Biotechnology Cat# sc-5279 RRID: AB_628051
Markers of the undifferentiated state (Immunocytochemistry)	Mouse IgG1 anti-NANOG	1:150	Santa Cruz Biotechnology Cat# sc-293121 RRID: AB_2665475
Markers of the undifferentiated state (Immunocytochemistry)	Mouse IgG3 anti-SSEA4	1:30	BioLegend Cat# 330402, RRID:AB_1089208
Differentiation markers (Immunocytochemistry)	Nestin-Alexa488	1:200	Technology Cat#33475. RRID:AB_2799037
Differentiation markers (Immunocytochemistry)	PAX6-Alexa647	1:200	Cell Signaling Technology Cat#60433. RRID:AB_2797599
Differentiation markers (Immunocytochemistry)	FOXA2-Alexa555	1:500	Cell Signaling Technology Cat#8186. RRID:AB_10891055
Differentiation markers (Immunocytochemistry)	GATA4-Alexa647	1:200	Cell Signaling Technology Cat#36966. RRID:AB_2799108Cell Signaling
Differentiation markers (Immunocytochemistry)	Brachyury-Alexa488	1:200	Cell signalling Technology Cat#81694. RRID:AB_2799983
Differentiation markers (Immunocytochemistry)	Vimentin-Alexa647	1:200	Cell signalling Technology Cat#5741. RRID:AB_10695459

Secondary antibodies (Immunocytochemistry)	Donkey anti-Mouse IgG (H+L) Highly cross-adsorbed Secondary, Alexa Fluor 488	1:500	Molecular Probes Cat# A-21206, RRID:AB_2535792
Secondary antibodies (Immunocytochemistry)	Donkey anti-Goat Mouse IgG (H+L) Highly cross-adsorbed Secondary, Alexa Fluor 647	1:500	Thermo Fisher Scientific Cat# A-11031, RRID:AB_144696
Secondary antibodies (Immunocytochemistry)	Goat anti-Mouse IgG3 Cross-adsorbed Secondary Antibody, Alexa Fluor 488	1:250	Thermo Fisher Scientific Cat# A-21151, RRID:AB_2535784
Secondary antibodies (Immunocytochemistry)	Goat anti-Mouse IgG1 Cross-adsorbed Secondary Antibody, Alexa Fluor 568	1:500	Thermo Fisher Scientific Cat# A-21124, RRID:AB_2535766
Secondary antibodies (Immunocytochemistry)	Goat anti-Mouse IgG2b Cross-adsorbed Secondary Antibody, Alexa Fluor 647	1:250	Thermo Fisher Scientific Cat# A-21242, RRID:AB_2535811

Site-specific nuclease		
Nuclease information	Nuclease type/ version	Alt-R Cas9-RNP
Delivery method	Electroporation	Nean Transfection System (program 6)
Selection strategy	Single cell cloning + restriction site analysis	

Primers and Oligonucleotides used in this study		
	Target	Forward/Reverse primer (5'-3')
Correction confirmation (PCR/ sequencing)	APP exon 17	GCCCACCACTAATAACCATTC/ TTCAGGATCCCACATCAGAG
gRNA1 off-target site 1	WNT9B	AATTGGTGGGGATGGAGGTT/ TTCCAAAGTCCTGTTAGTGCCT
gRNA1 off-target site 2	ATG16L2	CGCCTTCCCTCTTCGGTTTG/ TGTATTGCCCTGGAGTAAGCC
gRNA1 off-target site 3	PTPRJ	ACGAGCCAGGCTATTTTTGGA/ GCATCGAGACAGCACCGATA
gRNA1 off-target site 4	XKR6	AGCTTTCTTTCCAGTCGCTGT/ CCGAATTATTGCGGTGGCTT

gRNA1 off-target site 5	RP11-659P15.1	AATTTTGGGAGCCAGGGAGTTT/ CTGTCCAGTGATGTGGAGGAAG
gRNA2 off-target site 1	GPR158	CACTGTAGTCACCATCCTCAGAC/ GGATAGAACAGTCACAGACAGAG
gRNA2 off-target site 2	RP11-246A10.1	TTGGGTGTGAGATACACTGCTA/ GTGTAGTTTGGCCCCTTACCC
gRNA2 off-target site 3	AMFR	CACGTCGTGGGGTAACATCTG/ TGGATTCTTCCCAAAAGGACTACC
gRNA2 off-target site 4	KIAA1598	ATTTGTGTGACTTATTGTCTGGG/ TATGTCGTCTGCAAGGTCCC
gRNA2 off-target site 5	HEATR5A	ACCACAGCTGGCAAGAGATT/ TCAGTGCTGCTGTGGTATGT
gRNA1		GGTGTTCTTTGCAcAAGATG
gRNA2		GTGTTCTTTGCAcAAGATGT
ssODN		AATTGTAAATTATATTGCATTTAGAAATTA AAATTCTTTTTCTTAATTTGTTTTCAAGGT GTTCTTTGCAgAAGACGTCGGTTCAAACA AAGGTGCAATCATTGGACTCATGGTGGGC GGTGTTGTCATAGCGACAGTGA

Materials and Methods

Ethical statement

This study was approved by the LUMC Medical Ethical Committee(MEC) and informed consent was obtained from the HCHWA-D patient (NL45478.058.13/P13.080).

Cell culture and mutation correction

For generation of the parental hiPSC line LUMCi005-A, see previous publication[4]. Cells maintained at at 37˚C and 5% CO2 and were passaged weekly by dissociating using Gentle Cell Dissociation Reagent (Stem Cell Technologies). For genetic repair $1*10^5$ LUMCi005-A cells were electroporated at passage 20 with the Alt-R Cas9-RNP complex and the ssODN (both IDT) using program 6 of the Neon Transfection System (Invitrogen) and subsequently cultured in 2 Synthemax II-SC (Corning)-coated wells of a 12-well plate in TESR-plus with CloneR2 (Stem Cell Technologies). For single cell cloning, 1000 cells were plated onto a Synthemax II-SC-coated 10 cm dish in TESR-plus with CloneR2. After 8-12 days hiPSC colonies were split into VN-coated 2x wells of a 96-well plate in TESR-plus. The region of interest was amplified by PCR using the Terra PCR Direct Polymerase Mix (TaKaRa) from DNA isolated from one well (QuickExtract solution, Lucigen). Successfully edited clones were identified by AatII enzyme (New England Biolabs) activity and confirmed by Sanger sequencing performed by the Leiden Genome Technology Centre (LGTC). Off-target analysis was performed by PCR amplification and Sanger sequencing of the five most likely off-target

sites within coding regions as predicted by CRISPOR (crispor.tefor.net; Fig S1)[6].

Immunofluorescence staining

hiPSCs at passage 10 were fixed in 2% PFA for 30 min, permeabilized with 0.1% Triton X-100, and blocked with 4% normal swine serum (NSS, DAKO) at room temperature (RT) for 1 h. Primary antibodies were added and incubated overnight at 4°C. After washing, secondary antibodies were added for 1 h at RT (Table 2). Nuclei were stained with DAPI, and coverslips were mounted using Mowiol (Merck Millipore). Images were acquired on a Leica TCS SP8 microscope or the EVOS M7000.

FACS staining

hiPSCs were dissociated at passage 15 with TrypLE for 5 min at 37°C. Samples were fixed for 15 min in 200 µl of Reagent A of the fix & perm kit (Thermo Fisher Scientific). The cells were washed once in FACS buffer (PBS without Ca^{2+} and Mg^{2+} with 0.5% BSA and 2 mM EDTA), resuspended in 80 µl of Reagent B with the conjugated antibodies (Table 2) and incubated in the dark for 60 min at RT. Subsequently, the samples were washed once and resuspended in FACS buffer before being measured on the MACSQuant VYB flow cytometer (Miltenyi Biotec). Analysis was performed using the FlowJo™ v.10.6.1.

In vitro trilineage differentiation

The ability of hiPSCs to differentiate into the three germ layers (ectoderm, mesoderm, and endoderm) was assessed using differentiation with the Trilineage differentiation kit (Stem Cell Technologies). The differentiation was done according to the manufacturer's instructions on hESC-qualified Matrigel (Corning) coverslips. After 5 or 7 days of culture cells were fixed using 2%PFA.

Karyotype analysis

hiPSCs at passage 9 were karyotyped by Cell Guidance systems (UK) and a total of 20 metaphases were counted.

Sequencing

Genomic DNA was isolated from hiPSCs using Quick Extract (Lucigen) according to the manufacturer's instructions. The genes of interest were amplified using Platinum™ Taq DNA Polymerase High Fidelity (Invitrogen) for mutation and off-target analysis using the primers listed in Table 2.

Mycoplasma test

All lines were tested for the absence of mycoplasma and other human pathogens at passage 9 using the IDEXX BioAnalytics PCR analysis. (Supplementary Material 1).

Acknowledgements

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Competing interests

Christine L. Mummery is co-founder of Pluriomics bv (now Ncardia bv).

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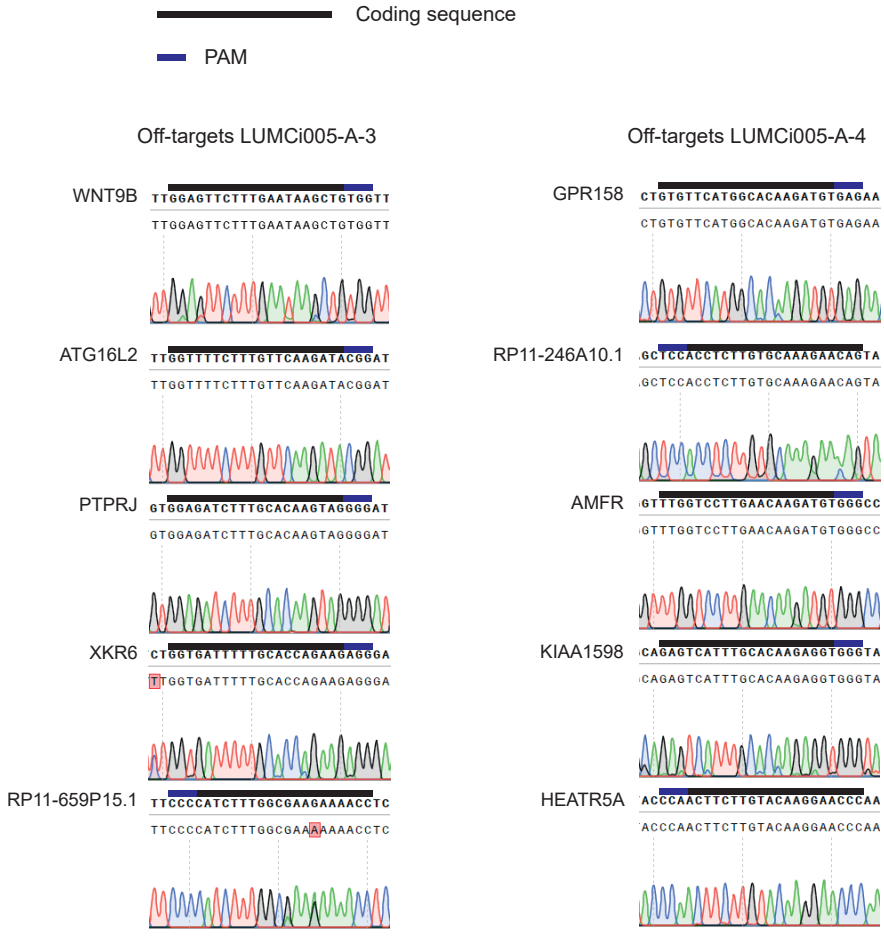
Supplementary Material

Contents

Supplementary Figure 1



Supplementary Figure 1: Sanger sequencing of potential off-targets

Supplementary Table 1: Mycoplasma test

	Measurement 1	Measurement 2	Ratio2/1	Mycoplasma
Positive control	70	2596	37.09	Positive
Negative control	138	32	0.231	Negative
LUMCi005-A-3	51	43	0.843	Negative
LUMCi005-A-4	51	33	0.647	Negative