



Lab Resource: Multiple Cell Lines

Generation of a heterozygous and a homozygous *CSF1R* knockout line from iPSC using CRISPR/Cas9

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ABSTRACT

Mutations in *Colony-stimulating factor 1 receptor (CSF1R)* lead to CSF1R-related leukoencephalopathy, also known as Adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP), a rapidly progressing neurodegenerative disease with severe cognitive and motor impairment. In this study, a homozygous and a heterozygous *CSF1R* knockout induced pluripotent stem cell (iPSC) line were generated by CRISPR/Cas9-based gene editing. These *in vitro* models will provide a helpful tool for investigating the still largely unknown pathophysiology of CSF1R-related leukoencephalopathy.

Resource Table:		(continued)	
Unique stem cell lines identifier	HIHCNi007-A-1 https://hpscereg.eu/cell-line/HIHCNi007-A-1 HIHCNi007-A-2 https://hpscereg.eu/cell-line/HIHCNi007-A-2	Type of Genetic Modification	CRISPR-Cas9-mediated gene knockout
Alternative name(s) of stem cell lines	iPSC-CSF1R ^{-/-} (HIHCNi007-A-1) iPSC-CSF1R ^{+/-} (HIHCNi007-A-2)	Associated disease	CSF1R-related leukoencephalopathy/Adult-onset leukoencephalopathy with axonal spheroids and pigmented glia, OMIM #221820
Institution	Hertie-Institute for Clinical Brain Research, University of Tübingen, and German Center for Neurodegenerative Diseases (DZNE), Germany	Gene/locus	CSF1R (GenBank: NM_005211.3)
Contact information of the reported cell line distributor	Stefanie Hayer stefanie.hayer@med.uni-tuebingen.de	Method of modification/site-specific nuclease used	Site specific nuclease CRISPR/Cas9
Type of cell lines	iPSC	Site-specific nuclease (SSN) delivery method	Nucleofection
Origin	Human	All genetic material introduced into the cells	crRNA1, crRNA2
Additional origin info (applicable for human ESC or iPSC)	Age: 74 Sex: male Ethnicity/breed/other genetic background information if known: Caucasian	Analysis of the nuclease-targeted allele status	Sequencing of the targeted allele
Cell Source	Fibroblasts	Method of the off-target nuclease activity surveillance	Sequencing of top 5 exonic off-targets
Method of reprogramming	Non-integrating episomal plasmids	Name of transgene	N/A
Clonality	Clonal	Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	N/A
Evidence of the reprogramming	PCR	Inducible/constitutive system details	N/A
transgene loss (including genomic copy if applicable)		Date archived/stock date	March 2021
Cell culture system used	Matrigel, Essential-8 medium, 37 °C, 5% CO ₂	Cell line repository/bank	https://hpscereg.eu/cell-line/HIHCNi007-A-1

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Table 1
Characterization and validation.

Classification (optional <i>italicized</i>)	Test	Result	Data
Morphology	Photography	normal	Bright-Field image (not shown, available on request)
Pluripotency status evidence for the described cell line	Qualitative analysis (Immunocytochemistry)	Expression of pluripotency markers TRA1-81, OCT4, Alkaline phosphatase expression	Fig. 1 panel B and E
	Quantitative analysis (RT-qPCR)	Expression of <i>OCT4</i> , <i>NANOG</i> , <i>KLF4</i> , <i>C-Myc</i> , <i>SOX2</i> , <i>DNMT3B</i> and <i>TDGF1</i>	Fig. 1 panel D
Karyotype	SNP array	No larger chromosomal aberrations or copy number variations upon CRISPR/Cas9 mediated genome editing	Fig. 1 panel F
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site	homozygous/heterozygous status confirmed	Fig. 1 panel A
	Transgene-specific PCR	N/A	N/A
Verification of the absence of random plasmid integration events	PCR	PCR	Data not shown, available on request
Parental and modified cell line genetic identity evidence	SNP array analysis	iPSC-CO, iPSC-CSF1R ^{-/-} and iPSC-CSF1R ^{+/-} matched	Submitted in archive with journal
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR or RT-PCR product)	Sanger sequencing homozygous/heterozygous 136 bp deletion	Fig. 1 panel A
	PCR-based analyses	N/A	N/A
	Southern Blot or WGS; western blotting (for knock-outs, KOs)	N/A	N/A
Off-target nuclease analysis-	PCR and sequencing across top 5 predicted off-target sites	No off-targets detected	Data not shown, available on request
Specific pathogen-free status	Mycoplasma	Mycoplasma testing by RT-PCR, negative	Data not shown, available on request
Multilineage differentiation potential	Embryoid body formation	Smooth muscle actin (SMA), β III-tubulin (TUJ) and FOXA2, SOX17	Fig. 1 panel C
Donor screening (OPTIONAL)	HIV 1 + 2, Hepatitis B, Hepatitis C	Not performed	
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping	Not performed	
	HLA tissue typing	Not performed	

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Ethical/GMO work approvals	https://hpscereg.eu/cell-line/HIHC-Ni007-A-2
	Ethics Institutional Review Board of the Medical Faculty, University of Tübingen, approval number 199/2011BO1
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	N/A

Resource utility.

Mutations in colony stimulating factor 1 receptor (*CSF1R*) are associated with *CSF1R*-related leukoencephalopathy (Rademakers et al., 2012). The underlying pathomechanism of this early-onset dementia is still largely unknown. Homozygous and heterozygous *CSF1R*-knockout iPSC lines will allow to further investigate the disease mechanisms in a human *in vitro* cell model.

1. Resource details

CSF1R-related leukoencephalopathy is an early-onset dementia characterized by severe cognitive and motor impairment, leading to death within 6–7 years (Papapetropoulos et al., 2022). It is caused by autosomal-dominant mutations in the *CSF1R* gene (Rademakers et al., 2012). *CSF1R* is a receptor tyrosine kinase mainly expressed in cells of hematopoietic origin, including monocytes, macrophages, and microglia. Mutations in *CSF1R*-related leukoencephalopathy lead to impairment of autophosphorylation and downstream signaling of the receptor.

Isogenic knockout cell lines of induced pluripotent stem cell (iPSC) lines are a valuable tool to study disease mechanisms on identical genetic backgrounds with the control cell line of origin. Here, we created a heterozygous and a homozygous knockout of *CSF1R* from a healthy control iPSC line (Table 1). Fibroblasts were obtained by skin biopsy

from a healthy 74-year-old male donor; the derived cells were nucleofected with episomal plasmids encoding hOCT4, hSOX2, hKLF4, hL-MYC and hLIN-28. The resulting iPSC were assessed for pluripotency and expanded for several passages. Subsequently, a CRISPR/Cas9-based knockout of *CSF1R* was performed by nucleofecting the iPSC with two fluorescence-labelled tracrRNA/crRNA/RNP complexes targeting exon 2, aiming to excise a 136 bp segment including the ATG region (Fig. 1A). Double-positive cells containing both crRNAs were selected via fluorescence-activated cell sorting (FACS) and seeded as single cells. Resulting colonies were picked, expanded and PCR-screened for homozygous or heterozygous knockout of *CSF1R*. The knockout state (Fig. 1A) as well as the absence of modifications in the 5 top *in silico* predicted exonic off-targets for each crRNA were confirmed by targeted Sanger sequencing. One heterozygous clone carrying the 136 bp deletion in the *CSF1R* gene on one allele (HIHCNi007-A-1) and one homozygous clone with the deletion on both alleles (HIHCNi007-A-2) were obtained. Heterozygosity of the heterozygous knockout clone was confirmed by single cell dilution of the original clone followed by sequencing analysis of the subclones, which were all heterozygous (Supplementary Fig. 1).

Pluripotency of both iPSC lines was confirmed on protein level by immunocytochemical staining of pluripotency markers OCT3/4 and TRA1-81 (Fig. 1B) as well as by assessment of the expression of the surface marker alkaline phosphatase (AP) (Fig. 1E). On RNA level, the expression of pluripotency genes *OCT3/4*, *SOX2*, *KLF4*, *c-MYC*, *NANOG*, *DNMT3B* and *TDGF1* was evaluated to demonstrate pluripotency (Fig. 1D). Further confirmation of pluripotency was obtained by spontaneous differentiation of the iPSC into all three germ layers and immunostaining of endo-, meso- and ectodermal markers FOXA2, SOX17, SMA and TUJ (Fig. 1C). Genomic integrity of these clones was verified by whole genome SNP genotyping (Fig. 1F).

Taken together, we generated iPSC lines with a heterozygous and a

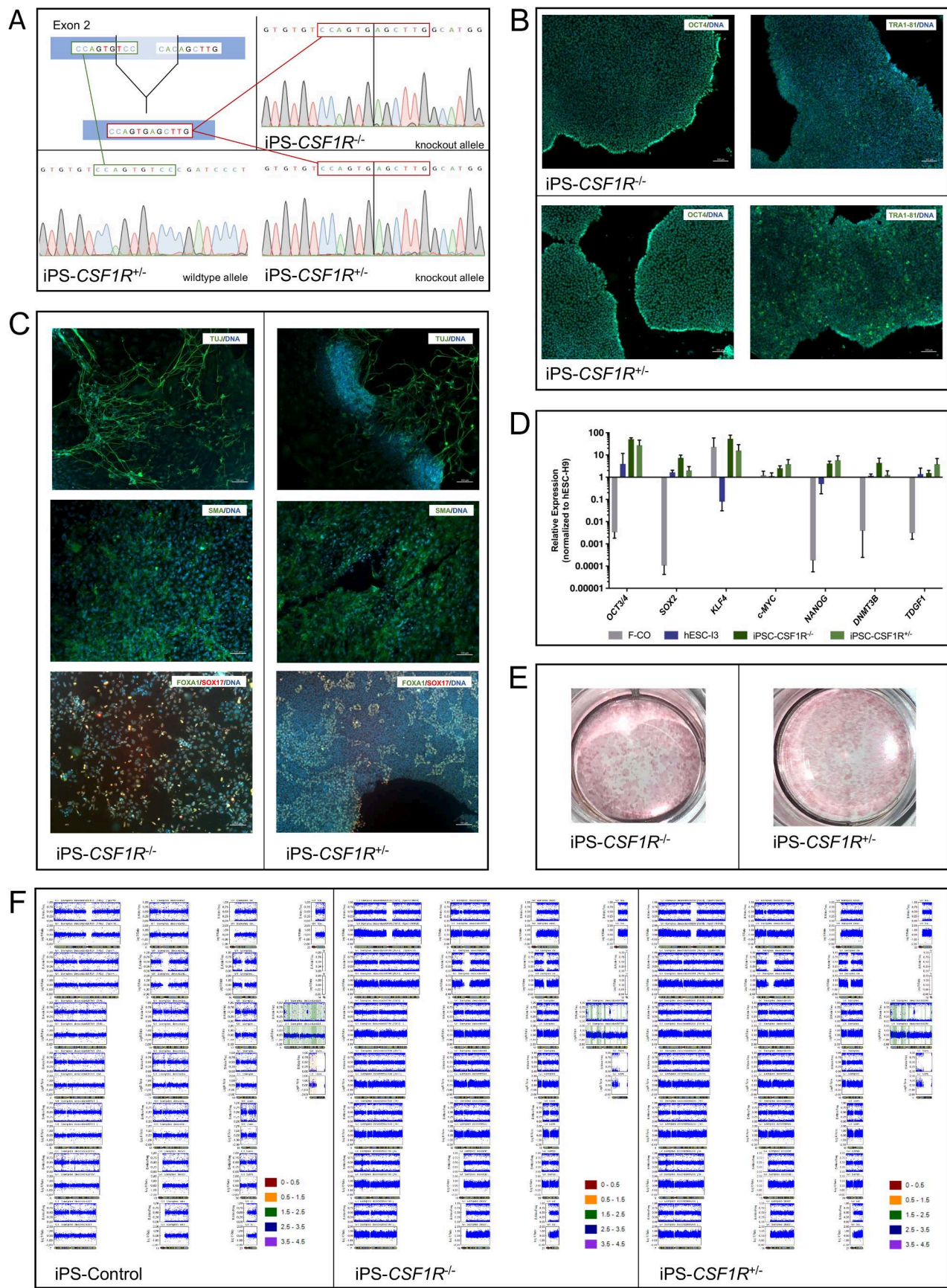


Fig. 1.

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry				
	Antibody	Dilution	Company Cat # and RRID	
Pluripotency Markers	Rabbit anti-OCT3/4	1:50	Proteintech, AB_2167545	
	Mouse anti-TRA1-81	1:500	Millipore, AB_177638	
In vitro Differentiation Markers	Rabbit anti-FOXA2	1:300	Millipore, AB_390153	
	Goat anti-SOX17	1:250	R&D Systems, AB_355060	
	Mouse anti-SMA	1:100	Dako, AB_2223500	
	Mouse anti-TUJ	1:1000	Sigma Aldrich, AB_477590	
Secondary antibodies	Alexa Fluor 488 Goat anti-rabbit IgG	1:1000	Life Technologies	
	Alexa Fluor 488 Goat anti-mouse IgG	1:1000	Life Technologies	
	Alexa Fluor 647 Donkey anti-goat IgG	1: 1000	Life Technologies	
Nuclear stain	DAPI	1:10000	Thermo Fisher Scientific, cat. # H3569	
Site-specific nuclease				
Nuclease information	Alt-R® S.p. Cas9 Nuclease V3			
Delivery method	Nucleofection			
Selection/enrichment strategy	FACS			
Primers and Oligonucleotides used in this study				
	Target	Forward/Reverse primer (5'-3')		
Episomal Plasmids (qPCR)	<i>KLF4</i>	CCACCTCGCCTTACACATGAAGA/ TAGCGTAAAAGGAGCAACATAG		
	<i>L-MYC</i>	GGCTGAGAAGAGGATGGCTAC/ TTTGTTTGACAGGAGCGACAAT		
	<i>OCT3/4</i>	CATTCAAACCTGAGGTAAGGG/ TAGCGTAAAAGGAGCAACATAG		
	<i>SOX2</i>	TTCACATGTCCCAGCACTACCAGA/ TTTGTTTGACAGGAGCGACAA		
	<i>c-MYC</i>	ATTCTCTGCTCTCCTCGACG/ CTGTGAGGAGGTTTGCTGTG		
	<i>DNMT3B</i>	ACGACACAGAGGACACACAT/ AAGCCCTTGATCTTTCCCA		
	<i>KLF4</i>	CCCCAAGATCAAGCAGGAGG/ GGGCAGGAAGGATGGGTAAT		
	<i>NANOG</i>	CAAAGGCAAACAACCCACTT/ TGCGTCACACCATTTGCTATT		
	<i>OCT4</i>	GGAAGGTATTGAGCCAAACG/ CTCCAGTTGCTCTCACTC		
		<i>SOX2</i>	TGATGGAGACGGAGCTGAAG/ GCTTGCTGATCTCCGAGTTG	
Pluripotency Markers (qPCR)	<i>TDGF1</i>	GGTCTGTGCCCCATGACA/ AGTTCTGGAGTCCTGGAAGC		
	<i>GAPDH</i>	TCACCAGGGCTGCTTTTAAC/ GACAAGCTTCCCGTTCTCAG		
		<i>CSF1R</i> Exon 2	TCTTCTCCCAAGACCCCTTGA/ GTCTAGTCTATCACTGTCCCCC	
		N/A		
		crRNA 1	CTCCGCAGGGATCGGGACAC TGGx	
		crRNA 2	TCCTGCTGGTGGCCACAGCT TGG	
		<i>CSF1R</i> Chromosome 5: 150.053.291–150.113.372	crRNA1: 150.086.500	
		PAM: NGG	crRNA2: 150.086.405	
		crRNA <i>CSF1R</i> Exon 2a <i>RP11-393I2.4/ZNF292</i>		
		<i>SLC43A1</i>	AAGTGATAGGGAGAGAGCC/ AGAAGGGTGCAGAGTTTCCA	
House-Keeping Genes (qPCR)		GGCTTGCTGATGATTCCAG/ ACTTCTGGTTTTCAAGCTCAGG		
		<i>CYTH2</i>	TGCATGCACGTGAACAAATG/ CCCATGTGCCTTTGGTGAAA	
		<i>AC068134.8/ALPP</i>	TTCAGGTTCACTGTTTTGC/ GCTACTCGGGAGGCTGAAG	
		<i>MINK1</i>	AGGGAATTGGGAAGTTGGGA/ TTGGGACTACAGGCATGCA	
		crRNA <i>CSF1R</i> Exon 2b <i>PLD4</i>	ACGCCATTACTTGCCATTGT/ GTCCTCTGCAAGCATCACAG	
		<i>ZNF407</i>	ACCTAGCAAGTCTACCCAGC/ CTGATCCTCCCTGCACTACC	
		<i>MATK</i>	CAGGCGCCTAGAGTCCTTAG/ GACCGAGAGAGTGGAGAGTG	
		<i>ANKDD1B</i>	CCCCTGACCACTCCACTATG/ CGTCCTTCCTTACCTCTCTCC	
		<i>BBS7</i>		

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Table 2 (continued)

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
ODNs/plasmids/RNA templates used as templates for HDR-mediated site-directed mutagenesis		GATGGGCACCTTGAACACTGT/ GAAGGGCAGACTCAGCAATTT	
		N/A	

homozygous *CSF1R* knockout, respectively. In addition to the patient-derived *CSF1R*-related leukoencephalopathy/ALSP iPSC line (Hayer et al., 2018), they are a valuable resource for an *in vitro* disease model for *CSF1R*-related leukoencephalopathy.

2. Materials and methods

2.1. CRISPR/Cas9-mediated knockout

The fully characterized iPSC line HIHCNi007-A (healthy control) was cultured on 1:60 Matrigel-coated plates with daily Essential 8 (E8) medium changes at 37 °C, 5% CO₂. At 70% confluency, cells were passaged 1:10 with 0.2% EDTA/PBS. At passage 10, 9 × 10⁵ cells were nucleofected with RNP-complexes containing two crRNAs (Table 2), a different ATTO-tracrRNA for each crRNA, and Cas9 (Integrated DNA technologies; Amaxa nucleofection system, Lonza, program B16). Cells positive for both markers were selected by FACS-sorting (SONY cell sorter SH800) and seeded in low density single-cell suspension (25000 cells/10 cm dish). After 10 days, colonies were picked, transferred to 24-well plates for expansion and PCR-screened for *CSF1R* knockout state. To confirm the heterozygous and homozygous knockout and exclude off target editing, Sanger sequencing analyses using specific primers (Table 2) were performed for the knockout locus and the top 5 exonic off-targets for both crRNAs targeted (3130xl Genetic Analyzer, Applied Biosystems).

2.2. Genomic integrity

DNA was isolated using the GeneJET Genomic DNA Purification Kit (Thermo Scientific). Whole genome SNP genotyping was conducted by Life & Brain GENOMICS using the Infinium OmniExpressExome-8-BeadChip (Illumina) and GenomeStudio V2.0.4. Copy number analysis was performed using the cnvPartition plugin (Illumina). Early mosaicism states were assessed by manual review on B allele frequency plots on chromosomal level. Integration of plasmids was excluded in the source iPSC line by means of PCR with plasmid-specific primers (Table 2).

2.3. Pluripotency assessment

Pluripotency was assessed at passage 10 + 6. Alkaline phosphatase expression was evaluated by fixing the cells with 4% paraformaldehyde for 1 min at 37 °C followed by 15–30 min incubation in 0.5 ml Fast Red (Sigma-Aldrich) with 0.03 ml Naphthol-AS-MX phosphate (Sigma-Aldrich). For immunostaining cells were cultured on coverslips, fixed as described above for 15 min, blocked for 1 h in BSA, and stained with the primary antibody (Table 2) overnight at 4 °C followed by incubation with the secondary antibodies 1 h at RT and counterstaining with 1 µg/ml DAPI for 20 min at RT (Table 2). Images were taken at 20x with the Axio Observer Z1.

To confirm expression of pluripotency transcripts and compare the levels to hESCs (H9), RNA was isolated using RNeasy Kit (Qiagen) and

transcribed to cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). RT-PCR was performed in triplicates using cDNA at a concentration of 1.25 ng/µl, SYBR Select Master Mix (Applied Biosciences) and specific primers (Table 2).

Spontaneous differentiation into all three germ layers was assessed with an embryoid body (EB)-based protocol using 1.2 × 10⁶ iPSC seeded in Aggrewell plates (Stem cell technologies) in EB medium (DMEM/F-12, 20% KO-SR, 1x Non-essential-amino-acid solution, 1% Penicillin-Streptomycin, 1% GlutaMAX, 0.0035% 50 µM β-Mercaptoethanol). On day 4 EBs were collected and plated on coverslips. Immunohistochemistry for TUJ and SMA was done after 10 days as described above. For endodermal differentiation, 2 × 10⁵ cells were seeded onto coverslips, cultured in endoderm induction medium (RPMI 1640 advanced, 1x B27, 1% Penicillin-Streptomycin, 0.2% FCS, 2 µM CHIR-99021, 50 ng/ml Activin A) for 4 days and stained for FOXA2 and SOX17.

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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cDNA of the hESC line I3 and H9 was kindly provided by the Institute of Reconstructive Neurobiology, Bonn, Germany. We acknowledge support by the Open Access Publishing Fund of the University of Tübingen.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2023.103066>.

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