Stem Cell Research 49 (2020) 102059

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Lab resource: Stem Cell Line

Generation of the CRISPR/Cas9-mediated KIF1C knock-out human iPSC line HIHRSi003-A-1

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A B S T R A C T

Bi-allelic loss-of-function mutations in the gene encoding the motor protein KIF1C are associated with Hereditary Spastic Paraplegia (HSP) type SPG58, a slowly progressive neurodegenerative motoneuron disease. The biological role of KIF1C is incompletely understood. We used a protein-based CRISPR/Cas9 genome editing approach to generate a homozygous KIF1C knock-out iPSC line (HIHRSi003-A-1) from a healthy control. This iPSC-KIF1C$^{-/-}$ line and the corresponding isogenic control are a useful model to study the physiological function of KIF1C and the pathophysiological consequences of KIF1C dysfunction in human disease.

1. Resource utility

Loss-of-function mutations in the gene encoding the microtubule dependent motor protein KIF1C are associated with Hereditary Spastic Paraplegia (Caballero Oteyza et al., 2014). Generation of functional KIF1C knock-out iPSCs and the corresponding isogenic control will allow to study the biological function of KIF1C and the pathomechanism of KIF1C-deficiency in disease-relevant cell types.

2. Resource details

Human skin fibroblasts from a healthy 24 year old female donor were reprogrammed using episomal plasmids expressing human OCT4, KLF4; L-MYC (OSKM), SOX2 and LIN28 (Okita et al., 2011) and expanded for several passages.

The resulting iPSCs (HIHRSi003-A (iPSC-CO)) were then used to generate a homozygous KIF1C knock-out line (Resource Table) using a protein based CRISPR/Cas9 genome-editing approach. Firstly, iPSC-CO were nucleofected with two ribonucleoprotein (RNP) complexes targeting exon 2 and 3 of the *KIF1C* gene. Fluorescently labelled tracrRNA (Atto550) was utilized to allow selection of cells that successfully incorporated the RNP complexes via fluorescence-activated cell sorting (FACS). Then, after single cell seeding the colonies were picked manually, screened via PCR and expanded for several passages. Presence of the CRISPR/Cas9-induced homozygous 71 bp deletion of parts of exon 2 and 3 of the KIF1C gene was validated by Sanger sequencing on the genomic level (Fig. 1A). Gel electrophoresis and western blotting further confirmed loss of KIF1C protein expression (Fig. 1B).

Resource Table

Unique stem cell line identifier	HIHRSi003-A-1
Alternative name(s) of stem cell line	iPSC-KIF1C$^{-/-}$
Institution	Hertie Institute for Clinical Brain Research German Center for Neurodegenerative Diseases (DZNE)
Contact information of distributor	Rebecca Schüle Rebecca.schuele-freyer@uni-tuebingen.de
Type of cell line	Induced pluripotent stem cell (iPSC)
Origin	Human
Additional origin info	Age: 24 years Sex: female
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogramming	Non-integrating episomal plasmids
Genetic Modification	YES
Type of Modification	CRISPR/Cas9-mediated gene knock-out
Associated disease	Hereditary Spastic Paraplegia SPG58/SPAX2 (OMIN #611302)
Gene/locus	NM_006612.5 (*KIF1C*); c.[158_227del], p.[Asp53Alafs*83]
Method of modification	CRISPR/Cas9
Name of transgene or resistance	N/A

(*continued on next page*)

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https://doi.org/10.1016/j.scr.2020.102059
Received 12 August 2020; Received in revised form 25 September 2020; Accepted 15 October 2020
Available online 29 October 2020

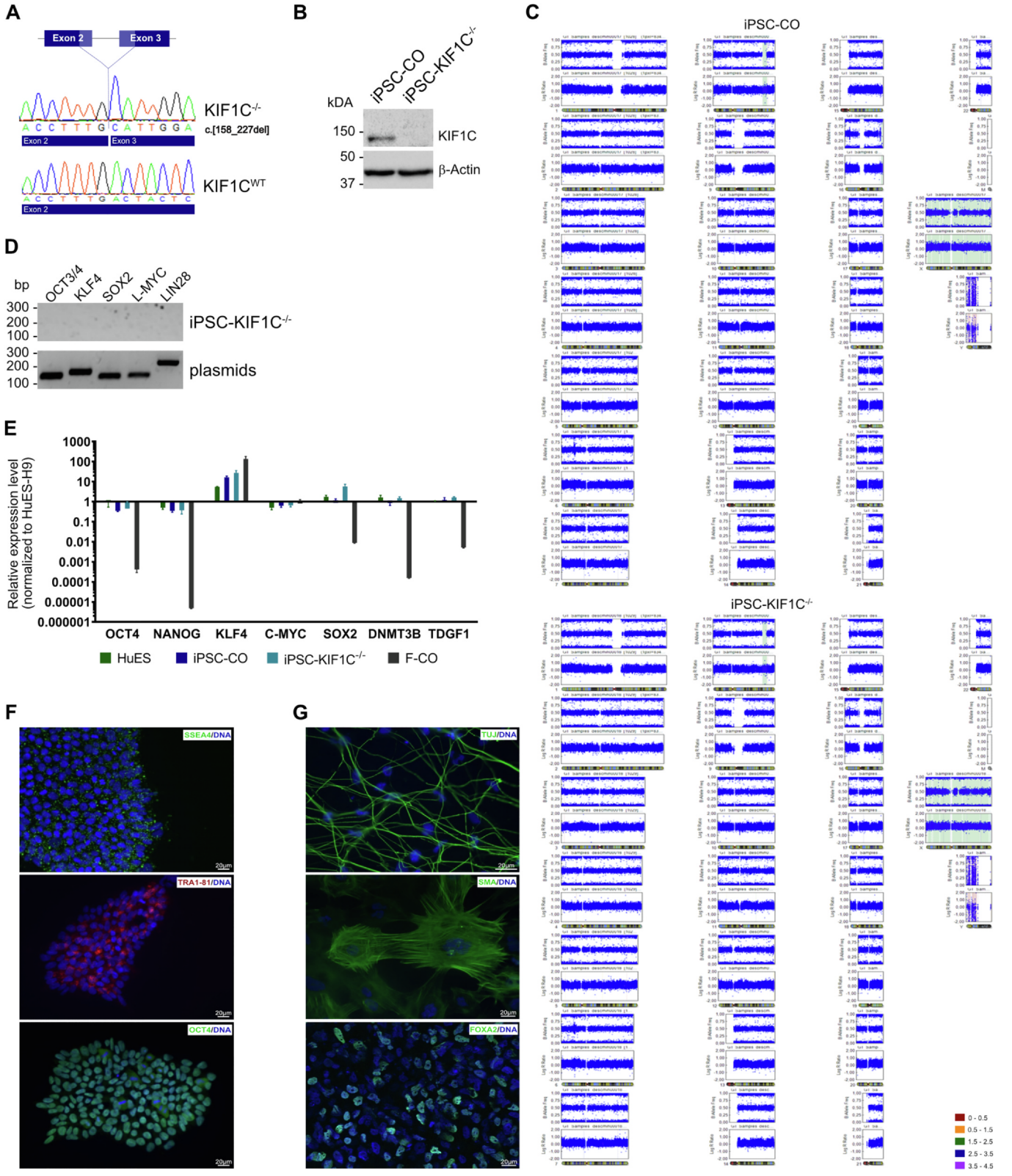

Fig. 1. Characterization and validation of HIHRSi003-A-1.

Resource Table (*continued*)

Inducible/constitutive system	N/A
Date archived/stock date	December 2019

Resource Table (*continued*)

Cell line repository/bank	N/A
Ethical approval	Institutional Review Board ("Ethikkommission") University of Tübingen Medical School, Germany, approval number 649/2019B02 (2019/09/30)

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	normal	Not shown Available on request
Phenotype	Qualitative analysis	Immunocytochemistry of pluripotency markers: SSEA4, TRA1-81, OCT4, Alkaline phosphatase staining	Fig. 1 panel F Not shown
	Quantitative analysis (RT-qPCR)	RT-qPCR for OCT4, NANOG, KLF4, C-Myc, SOX2, DNMT3B and TDGF1	Fig. 1 panel E
	Protein expression analysis	No remaining KIF1C expression in iPSC-KIF1C$^{-/-}$	Fig. 1 panel B
Genotype	Whole genome SNP genotyping with Infinium OmniExpressExome-8 BeadChip (Illumina) Spacing (kbp): Mean: 3,03; Median: 1,36	No larger chromosomal aberrations or copy number variations after CRISPR/Cas9-mediated genome editing	Fig. 1 panel C
Identity	STR analysis	5 sites: F-CO, iPSC-CO and iPSC-KIF1C$^{-/-}$; all genotypes match	Submitted in archive with the journal
Mutation analysis	Sequencing Southern Blot OR WGS	c.[158_277del], p.[Asp53Alafs*83] N/A	Fig. 1 panel A
Microbiology and virology	Mycoplasma	Mycoplasma testing by RT-PCR, negative	Supplementary figure A
Differentiation potential	Embryoid body formation	ß-tubulin (TUJ), smooth muscle actin (SMA), FOXA2	Fig. 1 panel G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	
Genotype additional info (OPTIONAL)	Blood group genotyping HLA tissue typing	N/A N/A	

To confirm the genomic integrity of the iPSC-KIF1C$^{-/-}$ line whole-genome SNP genotyping (Fig. 1C) was performed. Additionally, the top six predicted potential off target-effects of Cas9 for both crRNAs were excluded via Sanger sequencing (Supplementary figure C, Supplementary file). The iPSC line was transgene-free (Fig. 1D) and mycoplasma-free (Supplementary figure A). Furthermore, it exhibited a normal embryonic stem cell like morphology (See Table 1).

Pluripotency was verified via the expression of pluripotency-associated surface markers (alkaline phosphatase) and with immunocytochemistry demonstrating the protein expression of markers characteristically expressed in stem cells (SSEA-4, TRA1-81 and OCT4; Fig. 1F). Furthermore, the transcriptional expression of OCT4, NANOG, KLF4, C-MYC, SOX2, DNMT3B and TDGF1 was analysed by RT-qPCR (Fig. 1E). Hereby, the iPSC-KIF1C$^{-/-}$ line has an expression pattern similar to human embryonic stem cell lines (HuES), whereas the expression pattern is clearly distinct from fibroblasts.

To confirm the pluripotency of the generated iPSC-KIF1C$^{-/-}$ line *in vitro*, embryoid-body-based spontaneous differentiation into ectodermal, mesodermal and endodermal cell lineages was assessed and the corresponding markers TUJ, SMA and FOXA2 were stained (Fig. 1G).

3. Materials and methods

3.1. Reprogramming and cell culture

Fibroblasts were cultivated in DMEM high glucose media with 10% FCS (Life Technologies) at 37 °C/5% CO_2. For reprogramming, 10^5 fibroblasts were nucleofected with the plasmids pCXLE-hUL, pCXLE-hSK and pCXLE-hOCT4 (1 µg each) (Okita et al., 2011). The next day, media was supplemented with 2 ng/ml FGF2 (Peprotech). On day three media was changed to Essential 8 (E8) media with 100 µM sodium butyrate and then changed every other day. After 3 weeks, colonies were picked and expanded. Cryo-stocks were obtained using E8 media with 40% KO-SR (Life Technologies), 10% DMSO (Sigma-Aldrich) and 1 µM Y-27632 (Abcam Biochemicals). A PCR Mycoplasma Test (AppliChem) was used following manufacturer's recommendation.

3.2. Genome editing with CRISPR/Cas9

9 × 10^5 iPSC-CO cells were nucleofected with two pre-assembled RNP complexes containing Cas9 and crRNA-Atto550 tracrRNA (Table 2) (Integrated DNA Technologies). After FACS of Atto550 positive iPSCs, single cells were seeded, colonies were manually picked after 7–10 days and subsequently screened by PCR. DNA of iPSCs was isolated with GeneJET-Genomic DNA Purification Kit (Thermo Fisher Scientific). Presence of the homozygous deletion was confirmed by Sanger sequencing using specific primers (Table 2). To confirm loss of KIF1C protein, cells were lysed in RIPA Buffer (Sigma-Aldrich) and protein concentrations were measured with BCA-Protein Assay Kit (Thermo Fisher). After gel electrophoresis of 30 µg protein with 10% Bis-Tris gel and MOPS running buffer (Life Technologies), wet transfer was performed, and protein levels were detected with corresponding antibodies (Table 2).

3.3. Genomic integrity analysis

To exclude plasmid integration, RT-PCR was performed with plasmid specific primers (Table 2). The parental lineage of reprogrammed cells was performed by STR analysis of 5 loci (donor fibroblasts, derived iPSC-CO, iPSC-KIF1C$^{-/-}$). Top 6 off targets of each crRNA were analyzed with Sanger sequencing.

Whole-genome SNP genotyping was performed using Infinium OmniExpressExome-8-BeadChip (Illumina) to confirm genomic integrity.

3.4. Pluripotency analyses

Protein expression of pluripotency markers: Cells were fixed using 4% PFA and evaluated for alkaline phosphatase expression. Immunostaining was performed with fixed iPSC, specific antibodies (Table 2) and nuclei stain Hoechst 33,342 (1:10.000, Invitrogen) following standard protocols.

Transcript expression of pluripotency markers: RNA was extracted (High Pure RNA Isolation Kit, Roche) and reverse-transcribed to cDNA (Transcriptor First Strand cDNA Synthesis Kit, Roche) according to the manufacturer's protocol. RT-qPCR was performed in triplicates using Light Cycler 480 SYBR Green I Master (Roche). CT values were determined using the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH and the reference hESC line HuES-H9.

Embryoid-body-based spontaneous differentiation of iPSC-KIF1C$^{-/-}$ into all three germ layers: iPSCs were plated on AggreWell800 Plates (StemCell Technologies) in EB medium (80% DMEM/F12 (Life Technologies), 20% KO-SR, 1× NEAA (Sigma-Aldrich), 1× Penicillin-Streptomycin (Merck Millipore), 2 mM l-Glutamine (Gibco), 0.1 mM β-Mercaptoethanol (Merck)). Embryonic bodies were collected after 4 days and plated into matrigel-coated plates for ecto-, meso- and

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit anti-OCT4	1:100	Proteintech, AB_2167545
	Mouse anti-TRA1-81	1:500	Millipore, AB_177638
	Mouse anti-SSEA-4	1:500	Abcam, AB_778073
In vitro Differentiation Markers	Mouse anti-SMA	1:100	Dako, AB_2223500
	Rabbit anti-FoxA2	1:300	Millipore, AB_390153
	Mouse anti-TUJ	1:1000	Sigma Aldrich, AB_477590
Western Blotting	Rabbit anti-KIF1C	1:500	Abcam AB_1269252
	Mouse anti-ß-Actin	1: 10,000	Sigma Aldrich AB_476744
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 568 Goat anti-mouse IgG	1:1000	Life Technologies
	Peroxidase-conjugated AffiniPure Goat anti-rabbit	1:10,000	Jackson ImmunoResearch
	Peroxidase-conjugated AffiniPure Goat anti-mouse	1:10,000	Jackson ImmunoResearch
Primers			
	Target	**Forward/Reverse primer (5′-3′)**	
Episomal Plasmids (PCR)	OCT3/4_Plasmid	CATTCAAACTGAGGTAAGGG/TAGCGTAAAAGGAGCAACATAG	
	SOX2_Plasmid	TTCACATGTCCCAGCACTACCAG/TTTGTTTGACAGGAGCGACAAT	
	KLF4_Plasmid	CCACCTCGCCTTACACATGAAG/TAGCGTAAAAGGAGCAACATAG	
	L-MYC_Plasmid	GGCTGAGAAGAGGATGGCTAC/TTTGTTTGACAGGAGCGACAAT	
	LIN28_Plasmid	AGCCATATGGTAGCCTCATGTCCGC/ TAGCGTAAAAGGAGCAACATAG	
Pluripotency Markers (qPCR)	OCT4	GGAAGGTATTCAGCCAAACG/CTCCAGGTTGCCTCTCACTC	
	SOX2	AGCTCGCAGACCTACATGAA/CCGGGGAGATACATGCTGAT	
	KLF4	CCCCAAGATCAAGCAGGAGG/GGGCAGGAAGGATGGGTAAT	
	C-MYC	ATTCTCTGCTCTCCTCGACG/CTGTGAGGAGGTTTGCTGTG	
	NANOG	CAAAGGCAAACAACCCACTT/TGCGTCACACCATTGCTATT	
	DNMT3B	ACGACACAGAGGACACACAT/AAGCCCTTGATCTTTCCCCA	
	TDGF1	GGTCTGTGCCCCATGACA/AGTTCTGGAGTCCTGGAAGC	
House-Keeping Gene (qPCR)	GAPDH	TCACCAGGGCTGCTTTTAAC/GACAAGCTTCCCGTTCTCAG	
Sequencing of KIF1C KO	KIF1C Exon2-3	CGGGTCCTAGGAAGCCAAAAT/ CTGTTCCATAATCCTCCGACCC	
CRISPR Guide RNAs			
	Target	**Sequence (5′-3′)**	
crRNAs	KIF1C Exon 2	TGACCAGTAGGAGTAGTCAA	
	KIF1C Exon 3	CAGCAAGTGTATCGGGACAT	

endodermal differentiation. Differentiation was validated via immunostainings using the corresponding markers TUJ, SMA and FOXA2 (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.

Acknowledgements

cDNA of the human embryonic cell lines was kindly provided by the Institute of Reconstructive Neurobiology, Bonn, Germany. We thank Jennifer Reichbauer for excellent technical assistance.

This work was supported by the German Center for Neurodegenerative Diseases (DZNE; funding to R.S.), the Bundesministerium für Bildung und Forschung (BMBF) through funding for the TreatHSP network (01GM1905 to R.S.), the European Union's Horizon 2020 research and innovation programme under grant agreement No 779257 for the Solve-RD project (R.S.) and the National Institute of Neurological Disorders and Stroke and the National Institutes of Health under Award Number 5R01NS072248 (R.S.)

Authors of this publication are members of the European Reference Network for Rare Neurological Diseases – Project ID No 739510 (R.S., L.S.). We acknowledge support by Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of University of Tübingen.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2020.102059.

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