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Lab Resource: Stem Cell Line

Establishment of SPAST mutant induced pluripotent stem cells (iPSCs) from a hereditary spastic paraplegia (HSP) patient

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ABSTRACT

Human skin fibroblasts were isolated from a 40-year-old hereditary spastic paraplegia patient carrying an intronic splice site mutation (c.1687 + 2 T > A) in *SPAST*, leading to hereditary spastic paraplegia type 4 (SPG4). Fibroblasts were reprogrammed using episomal plasmids carrying *hOCT4*, *hSOX2*, *hKLF4*, *hL-MYC* and *hLIN28*. The generated transgene-free line iPS-SPG4-splice retained the specific mutation with no additional genomic aberrations, expressed pluripotency markers and was able to differentiate into cells of all germ layers *in vitro*. The generated iPS-SPG4-splice line might be a useful platform to study the pathomechanism of SPG4.

Resource table:

Name of Stem Cell line	iPS-SPG4-splice
Institution	German Center for Neurodegenerative Diseases (DZNE), Tuebingen, Germany
Person who created resource	Stefan Hauser, Melanie Erzler, Yvonne Theurer, Stefanie Schuster
Contact person and email	Ludger Schöls; Ludger.Schoels@uni-tuebingen.de
Date archived/stock date	July 2015
Origin	Human skin fibroblasts
Type of resource	Biological reagent: induced pluripotent stem cell (iPSCs); derived from a SPG4 patient carrying a heterozygous c.1687 + 2 T > A splice site mutation
Sub-type	Induced pluripotent stem cells (iPSCs)
Key transcription factors	*hOCT4*, *hSOX2*, *hKLF4*, *hL-MYC*, *hLIN28* (Addgene plasmids 27,076, 27,078 and 27,080; Okita et al., 2011)
Authentication	Identity and purity of iPS-SPG4-splice line confirmed by analysis of plasmid integration, mutation sequencing, SNP array analysis, pluripotency markers and *in vitro* differentiation potential
Link to related literature	N/A
Information in public databases	N/A
Ethics	Patient informed consent obtained/ Ethics Review Board-competent authority approval obtained

1. Resource details

Hereditary spastic paraplegia (HSP) is a neurodegenerative disorder characterised by lower limb spasticity and weakness due to axonal degeneration in the corticospinal tract. The most common form of HSP is the autosomal dominantly inherited spastic paraplegia type 4 (SPG4) which is caused by mutations within the *SPAST* gene encoding for the microtubule-severing enzyme spastin (Schule et al., 2016). To study the underlying disease mechanisms, the iPSC line iPS-SPG4-splice was generated by delivery of episomal plasmids encoding human *OCT4*, *SOX2*, *KLF4*, *L-MYC* and *LIN28* in skin fibroblasts from a 40-year-old patient with a phenotype of pure HSP (Okita et al., 2011). Gait difficulties developed early in childhood with a slowly progressive course of disease. The quality of the generated iPSCs was investigated by genotypic and functional assays. Genomic integrity was analysed by comparative SNP analysis of fibroblasts and the generated iPSCs (Fig. 1A), resequencing of the mutation-site (Fig. 1B) and exclusion of genomic integration of episomal plasmids (Fig. 1C). The expression of pluripotency markers on protein and RNA level was assessed by alkaline phosphatase staining (ALP) (Fig. 2A), immunocytochemical stainings of OCT4, TRA-

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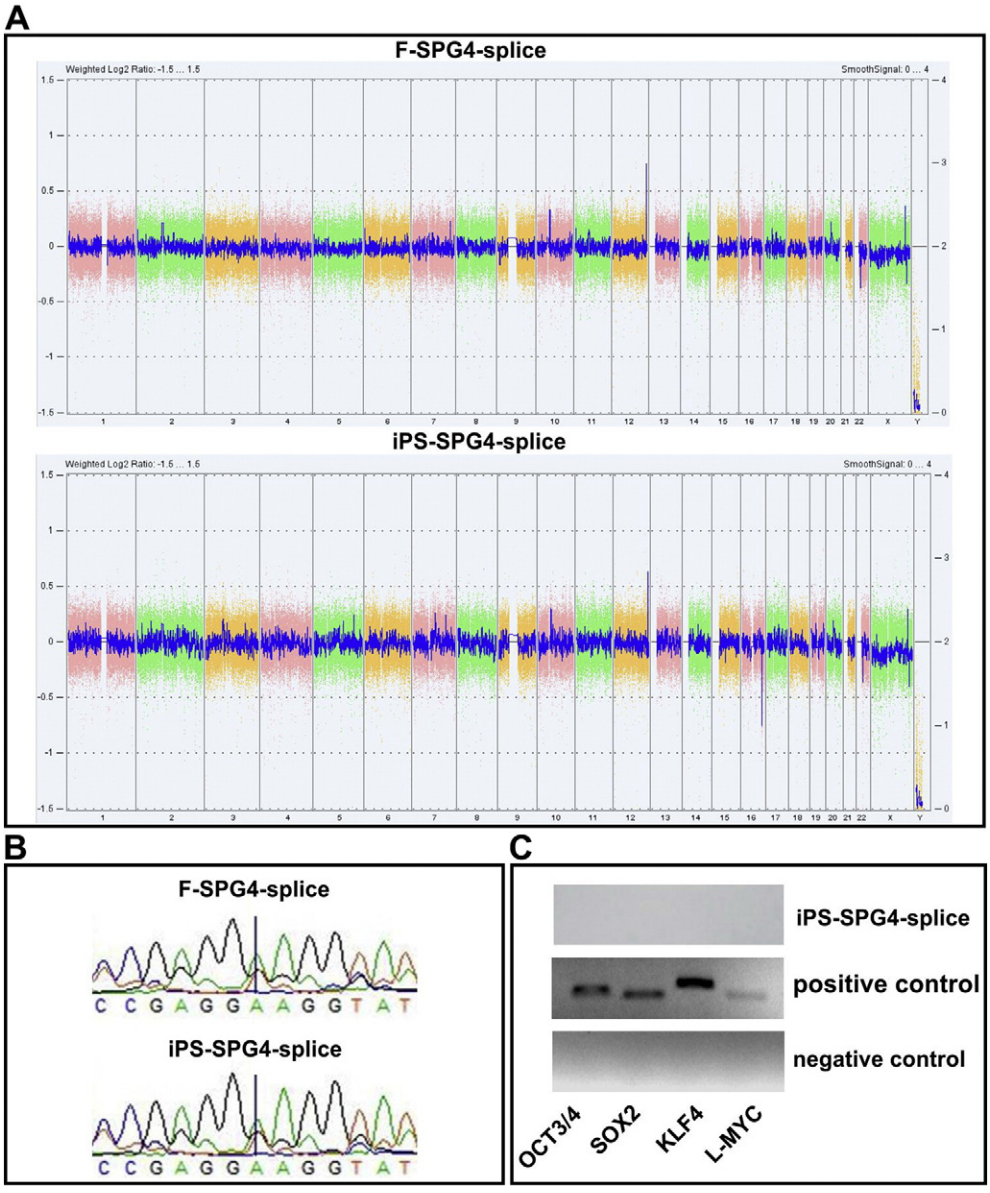

Fig. 1. Genomic characterisation of generated iPS-SPG4-splice. (A) SNP array analysis of fibroblast (F-SPG4-splice) and generated iPSCs (iPS-SPG4-splice) reveal genomic integrity. Data is shown in whole genome view (WGV) and expressed as the weighted log2 ratio of the copy number on the left Y-axis (blue line), and the chromosome number on the X-axis. (B) Sanger sequencing of the region containing the heterozygous splice site mutation c.1687 + 2 T > A in F-SPG4-splice and iPS-SPG4-splice. (C) RT-PCR verification of the absence of plasmid integration in iPS-SPG4-splice. Plasmid-specific primer for hOCT3/4, SOX2, KLF4, and L-MYC with DNA from iPS-SPG4-splice, plasmid samples as positive control, and ddH_2O as negative control were used.

1-81, NANOG and SSEA-4 (Fig. 2B) as well as qRT-PCR analysis of OCT4, NANOG, KLF4, c-MYC, SOX2, REX1, DNMT3B and TDGF1 in comparison to human embryonic stem cell lines (HuES-H6 / HuES—H9) and fibroblasts (Fig. 2C). Additionally, the potential of generated iPSCs to differentiate into cells of all three germ layers was investigated. iPSCs were able to differentiate into neurons expressing β-III-tubulin, muscle cells positive for α-smooth muscle actin (SMA), and early endodermal cells positive for α-fetoprotein (AFP) (Fig. 2D).

2. Materials and methods

2.1. Reprogramming of fibroblast to iPSCs

Patient fibroblasts carrying a heterozygous splice site mutation (c.1687 + 2 T > A) in *SPAST* were derived from skin biopsies by dissection and cultivation in fibroblast medium consisting of Dulbecco's modified eagle's medium (DMEM) high glucose (Life technologies) with 10% fetal bovine serum (FBS, Life technologies). After approx. 10 days of cultivation at 37 °C and 5% CO_2 fibroblasts were collected and expanded by medium change every 2–3 days. Reprogramming was achieved by nucleofection of 1×10^5 cells with 1 μg of each plasmid (*hOCT4*, *hSOX2*, *hKLF4*, *hL-MYC* and *hLIN28* (Okita et al., 2011)) using the Nucleofector 2D system (Lonza). After reprogramming, cells were replaced in one well of a 6-well plate and cultivated for 1 day in fibroblast medium. After a period of 2 days in fibroblast medium supplemented with 2 ng/ml FGF-2 (Peprotech) cells were transferred to Essential 8 (E8) medium containing 100 μM NaB (Sigma-Aldrich). 3–4 weeks after reprogramming, iPSC colonies were picked manually and further cultivated on Matrigel-coated 6-well dishes using E8 medium. IPSCs were split in a ratio of 1:6–1:12 by adding PBS/EDTA (0.02% EDTA in

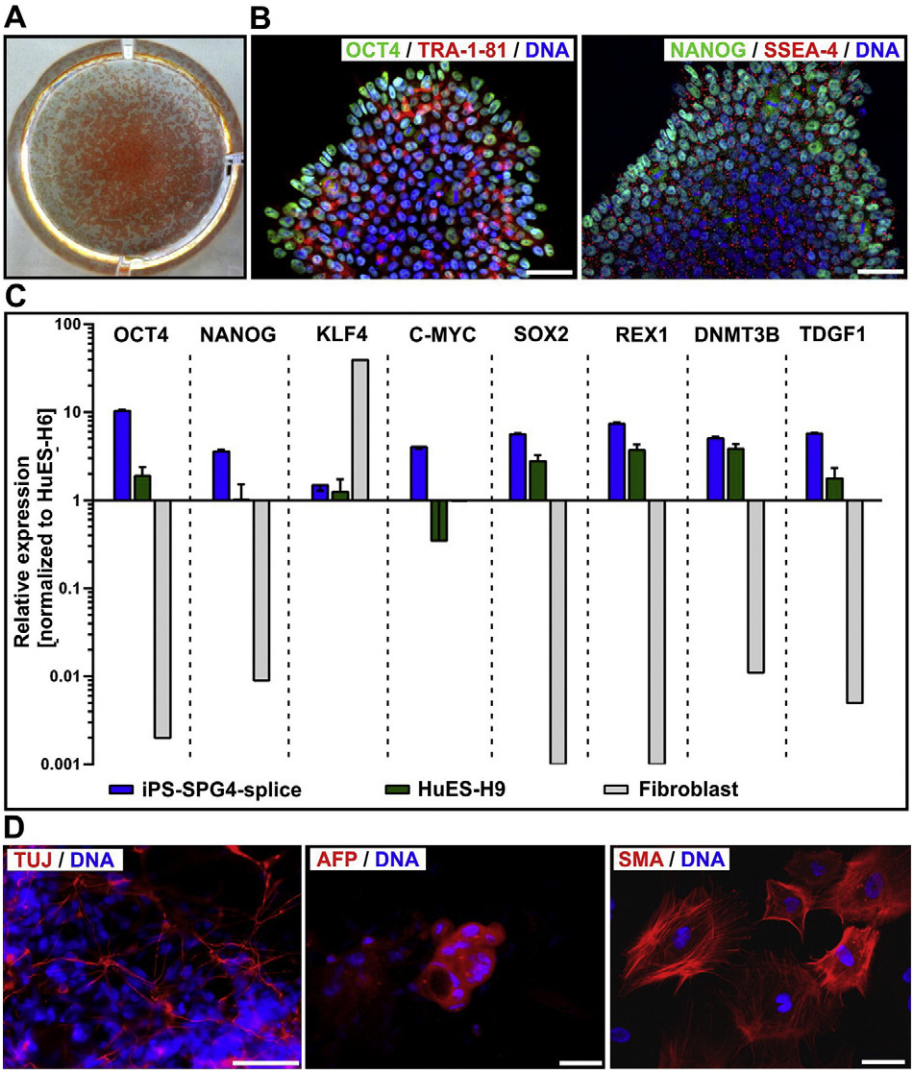

Fig. 2. Functional characterisation of generated iPS-SPG4-splice by (A) alkaline phosphatase (ALP) activity and the expression of specific pluripotency marker identified *via* (B) immunocytochemical staining of OCT4 (green), TRA1–81 (red) as well as NANOG (green) and SSEA-4 (red). Nuclei are counterstained with DAPI (blue). Scale bar = 50 μm. (C) qRT-PCR with cDNA from iPS-SPG4-splice, HuES—H6, HuES-H9 and fibroblasts and pluripotency specific primers OCT4, NANOG, KLF4, C-MYC, SOX2, REX1, DNMT3B and TDGF1 normalized to the housekeeping gene GAPDH and the hESCs HuES—H6. (D) Immunostainings of ectodermal (β-III-tubulin (TUJ), scale bar = 100 μm), endodermal (α-fetoprotein (AFP), scale bar = 50 μm) and mesodermal (α-smooth-muscle-actin (SMA), scale bar = 50 μm) markers reveal the differentiation potential of iPS-SPG4-splice. Nuclei are counterstained with DAPI (blue).

PBS). Between passage 5 and 10, cells were analysed and frozen in E8 medium with 40% KOSR (Life technologies), 10% DMSO (Sigma-Aldrich) and 1 μM Y-27632 (Abcam Biochemicals).

2.2. SNP array analysis

DNA was isolated using the DNeasy blood & tissue kit (Qiagen) according to the manufacturer's guidelines. Using the Affymetrix CytoScan HD technology (Affymetrix) SNP array analysis was performed by using 2 μg of DNA of the iPSC line as well as the original fibroblast line. Raw data was processed using Affymetrix Chromsome Analysis Suite (ChAS) 2.0 software.

Table 1
Primers (Okita et al., 2011) used for integration analysis by PCR.

	Forward sequence	Reverse sequence
KLF4	CCACCTCGCCTTACACATGAAG	TAGCGTAAAAGGAGCAACATAG
L-MYC	GGCTGAGAAGAGGATGGCTAC	TTTGTTTGACAGGAGCGACAAT
OCT3/4	CATTCAAACTGAGGTAAGGG	TAGCGTAAAAGGAGCAACATAG
SOX2	TTCACATGTCCCAGCACTACCAG	TTTGTTTGACAGGAGCGACAAT

2.3. Sequencing of mutation site

Using the 3130xl Genetic Analyzer (Applied Biosystems) and a primer set flanking the mutation (c.1687 + 2 T > A) in *SPAST*, the mutation site was analysed by applying standard procedures and visualization with Staden 2.0.0b10 software (Staden Sourceforge).

Table 2
Primers used for validation of pluripotency genes.

	Forward sequence	Reverse sequence
c-MYC	ATTCTCTGCTCTCCTCGACG	CTGTGAGGAGGTTTGCTGTG
DNMT3	ACGACACAGAGGACACACAT	AAGCCCTTGATCTTTCCCCA
GAPDH	AGGTCGGAGTCAACGGATTT	ATCTCGCTCCTGGAAGATGG
KLF4	CCATCTTTCTCCACGTTCGC	CGTTGAACTCCTCGGTCTCT
NANOG	CAAAGGCAAACAACCCACTT	TGCGTCACACCATTGCTATT
OCT4	GGAAGGTATTCAGCCAAACG	CTCCAGGTTGCCTCTCACTC
REX1	AACGGGCAAAGACAAGACAC	AACTCACCCCTTATGACGCA
SOX2	TGATGGAGACGGAGCTGAAG	GCTTGCTGATCTCCGAGTTG
TDGF1	GGTCTGTGCCCCATGACA	AGTTCTGGAGTCCTGGAAGC

Table 3
Antibodies used for validation of pluripotency genes and *in vitro* differentiation potential.

	Antibody	Dilution	Manufacturer
Pluripotency	rabbit *anti*-NANOG	1:50	Stemgent
	goat anti-OCT4	1:100	Santa-Cruz
	mouse anti-SSEA4	1:500	Abcam
	mouse anti-TRA-1-80	1:500	Merck-Millipore
In vitro differentiation	mouse anti-AFP	1:200	Sigma-Aldrich
	mouse anti-SMA	1:100	Dako
	mouse anti-TUJ	1:1000	Sigma-Aldrich

2.4. Non-integration of transgenes

DNA was isolated as described before. Using plasmid specific primers (see Table 1) as well as the three episomal plasmids (pCXLE-hUL, pCXLE-hSK and pCXLE-hOCT4) as positive control the integration was analysed. Therefore, RT-PCR reactions were performed using GoTaq G2 DNA Polymerase (Promega) according to the manufacturer's instruction. PCR products were separated on a 2.0% agarose gel and visualized with Midori Green.

2.5. Alkaline phosphatase staining

For ALP staining iPSCs were cultivated on 12-well plates and fixed in 4% paraformaldehyde (PFA) for 1 minute. After 3 times washing in PBS staining solution (40 µl Naphthol AS-MX phosphate alkaline solution (Sigma-Aldrich) and 1 ml Fast Red (1 mg/ml, Sigma Aldrich)) was added for 30 min. ALP-positive colonies were stained dark red.

2.6. Immunocytochemical staining

iPSCs were cultivated on 24-well plates on coverslips until confluency of 60–80%. After fixation in 4% paraformaldehyde (PFA) for 15 min cells were washed 3 times with PBS and incubated in blocking buffer (PBS, 1% FCS, 0.1% Triton X-100) for 45 min. Cells were stained for 1 h at room temperature (RT) with the primary antibody (see Table 3). After 3 washes in PBS, cells were incubated with the secondary antibody (Alexa488 or Alexa568 diluted 1:300 (Life technologies)) in the dark at RT for 1 h. Nuclear counterstaining was achieved by addition of DAPI (1:10,000) for 15 min at RT in the dark. After embedding in ProLong Diamond Antifade Mountant cells were analysed with Axio Imager Z1 with ApoTome (Zeiss).

2.7. qRT-PCR of pluripotency marker

RNA was isolated using the High Pure RNA Isolation Kit (Roche) according to the manufacturer's guidelines. Reverse transcription was performed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Using Light Cycler 480 SYBR Green I Master (Roche) and specific pluripotency gene primers (see Table 2) qRT-PCR was performed. Runs were performed as triplicates and C_T-values were normalized using the $2^{-\Delta\Delta Ct}$ method with the hESC line HuES-H6 as reference and GAPDH as housekeeping gene.

2.8. In vitro differentiation potential

For embryonic body (EB) generation iPSCs were cultivated in EB medium consisting of 80% DMEM/F12 (Life technologies), 20% KOSR, 1× NEAA, 1× Pen/Strep, 2 mM L-Glutamine and 0.1 mM 2-Mercaptoethanol on AggreWell 800 plates (Stemcell Technologies) with medium change at day 2. EBs were collected on day 4 and plated on 0.1% gelatine (Sigma-Aldrich) coated coverslips. Cells were cultivated for additional 2–3 weeks with medium change every other day and analysed by immunocytochemical staining using antibodies against AFP (α-fetoprotein), SMA (α-smooth muscle actin) and TUJ (β-III-tubulin) (see Table 3).

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