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Lab resource: Stem Cell Line

Generation of induced pluripotent stem cells (iPSCs) from a hereditary spastic paraplegia patient carrying a homozygous R486C mutation in CYP7B1 (SPG5)

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ABSTRACT

Skin fibroblasts were obtained from a 47-year-old hereditary spastic paraplegia patient carrying a homozygous mutation R486C in *CYP7B1* (Cytochrome P450, Family 7, Subfamily B, Polypeptide 1), responsible for causing hereditary spastic paraplegia type 5 (SPG5). Induced pluripotent stem cells (iPSCs) were generated by transfection with episomal plasmids carrying *hOCT4*, *hSOX2*, *hKLF4*, *hL-MYC* and *hLIN28*. The generated line iPS-SPG5-R486C was transgene-free, 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-SPG5-R486C line may be a useful resource for disease modelling of SPG5.

Resource table

Name of Stem Cell line	iPS-SPG5-R486C
Institution	German Center for Neurodegenerative Diseases (DZNE), Tuebingen, Germany
Person who created resource	Philip Höflinger, Stefan Hauser, Yvonne Theurer
Contact person and email	Stefan Hauser, stefan.hauser@dzne.de
Date archived/stock date	July 2015
Origin	Human skin fibroblasts
Type of resource	Biological reagent: induced pluripotent stem cell (iPSCs); derived from SPG5 patient with a homozygous *CYP7B1* mutation R486C
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-SPG5-R486C line confirmed by analysis of plasmid integration, mutation sequencing, SNP array analysis (Fig. 1), pluripotency markers and *in vitro* differentiation potential (Fig. 2)
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

Spastic paraplegia gene type 5 (SPG5) is an autosomal recessive subtype of hereditary spastic paraplegia caused by mutations in *CYP7B1*, a gene encoding for the cytochrome P-450 oxysterol 7-α-hydroxylase, essential for the liver-specific alternative pathway in bile acid synthesis (Schule et al., 2010). In this study, skin fibroblasts from a 47-year-old man were reprogrammed into iPSCs *via* electroporation of three episomal plasmids encoding human *OCT4*, *SOX2*, *KLF4*, *L-MYC* and *LIN28* (Okita et al., 2011). To determine the quality of generated iPSCs several genotypic and functional assays were performed. The characterization included analysis of genomic integrity given by comparative SNP analysis of parental fibroblasts and the respective iPSC line (Fig. 1A), resequencing of the mutation-site (Fig. 1B) and excluding the integration of episomal plasmids by PCR (Fig. 1C). Furthermore, the expression of stem cell marker on protein and RNA level were assessed by alkaline phosphatase staining (ALP) (Fig. 2A), immunocytochemical stainings of

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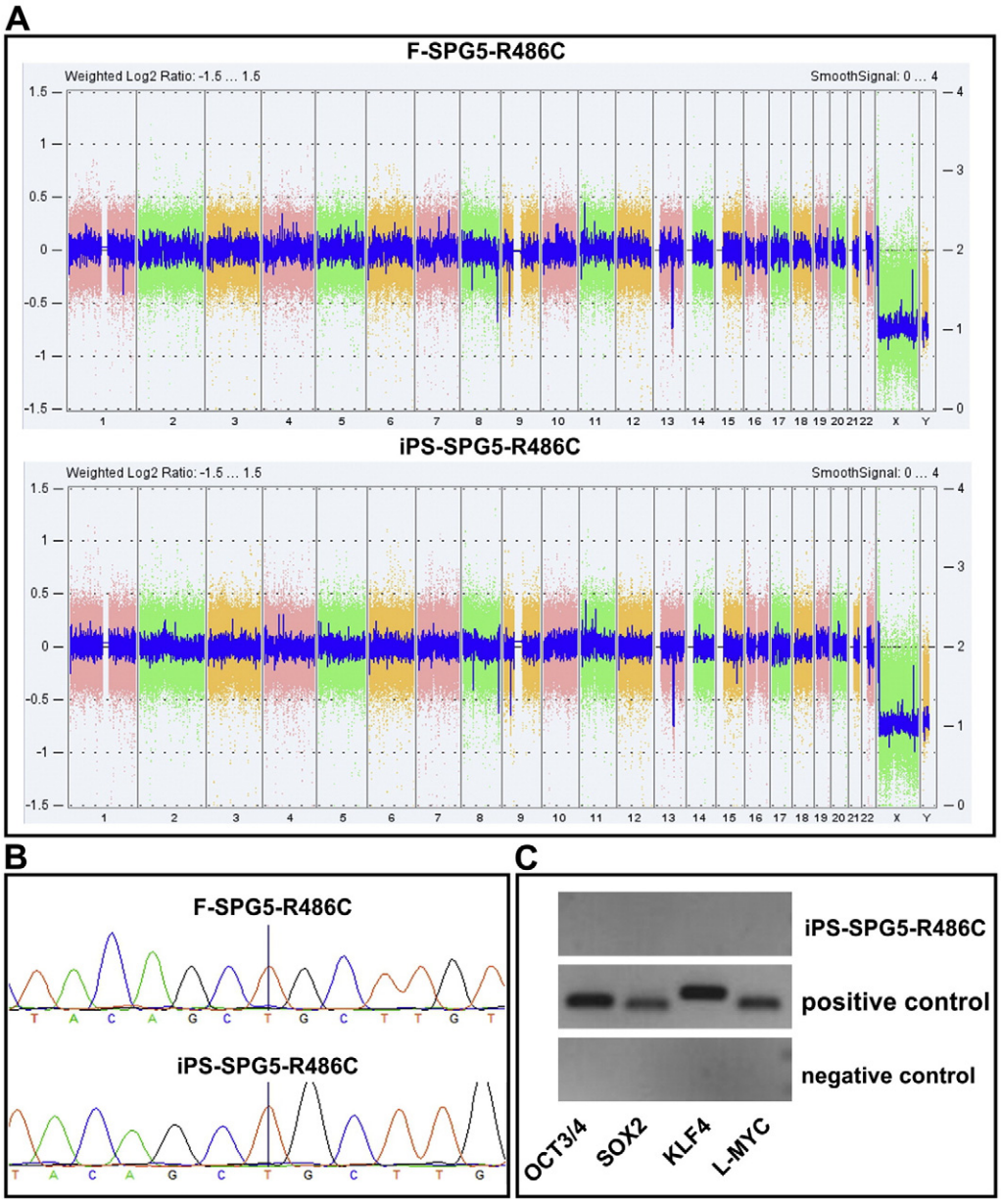

Fig. 1. Genomic validation of iPS-SPG5-R486C. (A) SNP array analysis of fibroblast (F-SPG5-R486C) and iPSCs (iPS-SPG5-R486C) reveal genomic integrity after reprogramming. Data is shown in whole genome view (WGV) and expressed as the weighted log2 ratio of the copy number on the left Y-axis (blue line), and the chromosome number on the X-axis. (B) Resequencing of patient-specific mutation. Electropherograms of sequences of F-SPG5-R486C and iPS-SPG5-R486C display homozygous point mutation c.1456C > T. (C) PCR analysis with DNA from iPS-SPG5-R486C, plasmid samples as positive control, and ddH_2O as negative control with plasmid-specific primer for OCT3/4, SOX2, KLF4, and L-MYC.

OCT4 and NANOG (Fig. 2B) and qRT-PCR analysis of OCT4, SOX2, KLF4, c-MYC, NANOG, DNMT3B and TDGF1 in comparison to human embryonic stem cell lines (HuES-H6, HuESH1 and HuES-H9) and fibroblasts (Fig. 2C). To prove the functionality of the generated iPSCs, iPSC-derived embryoid bodies (EBs) were differentiated into cells of all three germ layers. Generated iPSCs were able to differentiate into neurons expressing β-III-tubulin, muscle cells positive for α-smooth muscle actin (SMA), and early endodermal structures like hepatic precursors detectable by antibodies against α-fetoprotein (AFP) (Fig. 2D).

2. Materials and methods

2.1. Reprogramming of fibroblast to iPSCs

A skin biopsy was obtained from a 47-year-old man carrying a homozygous R486C mutation within *CYP7B1*. Loss of functions effects of the mutation were shown by a metabolic block of the CYP7B1 substrate 27-hydroxy-cholesterol (27-OHC) in blood and cerebrospinal fluid (CSF). Serum 27-OHC was elevated by 930 ng/ml in the patient compared to 89–243 ng/ml in healthy controls (Dzeletovic et al., 1995; Norlin et al., 2000) and 131–369 ng/ml in heterozygous mutation carriers (Rattay et al., 2016). The biopsy was dissected and left undistributed in fibroblast medium consisting of Dulbecco's modified eagle's medium (DMEM) high glucose (Life technologies) with 10% fetal bovine serum (FBS, Life technologies) for 10 days at 37 °C and 5% CO_2 to allow fibroblasts to grow out from the biopsy. Expansion of skin fibroblast was achieved by medium change every 2–3 days. For reprogramming 1×10^5 fibroblasts were electroporated (Nucleofector 2D, Lonza) with a total of 1 μg per plasmid carrying the sequences for *hOCT4*, *hSOX2*, *hKLF4*, *hL-MYC* and *hLIN28* (Okita et al., 2011) and cultured in fibroblast medium for 1 day. After 2 days of cultivation in fibroblast medium containing 2 μg/l FGF-2 (Peprotech) cells were transferred to Essential 8

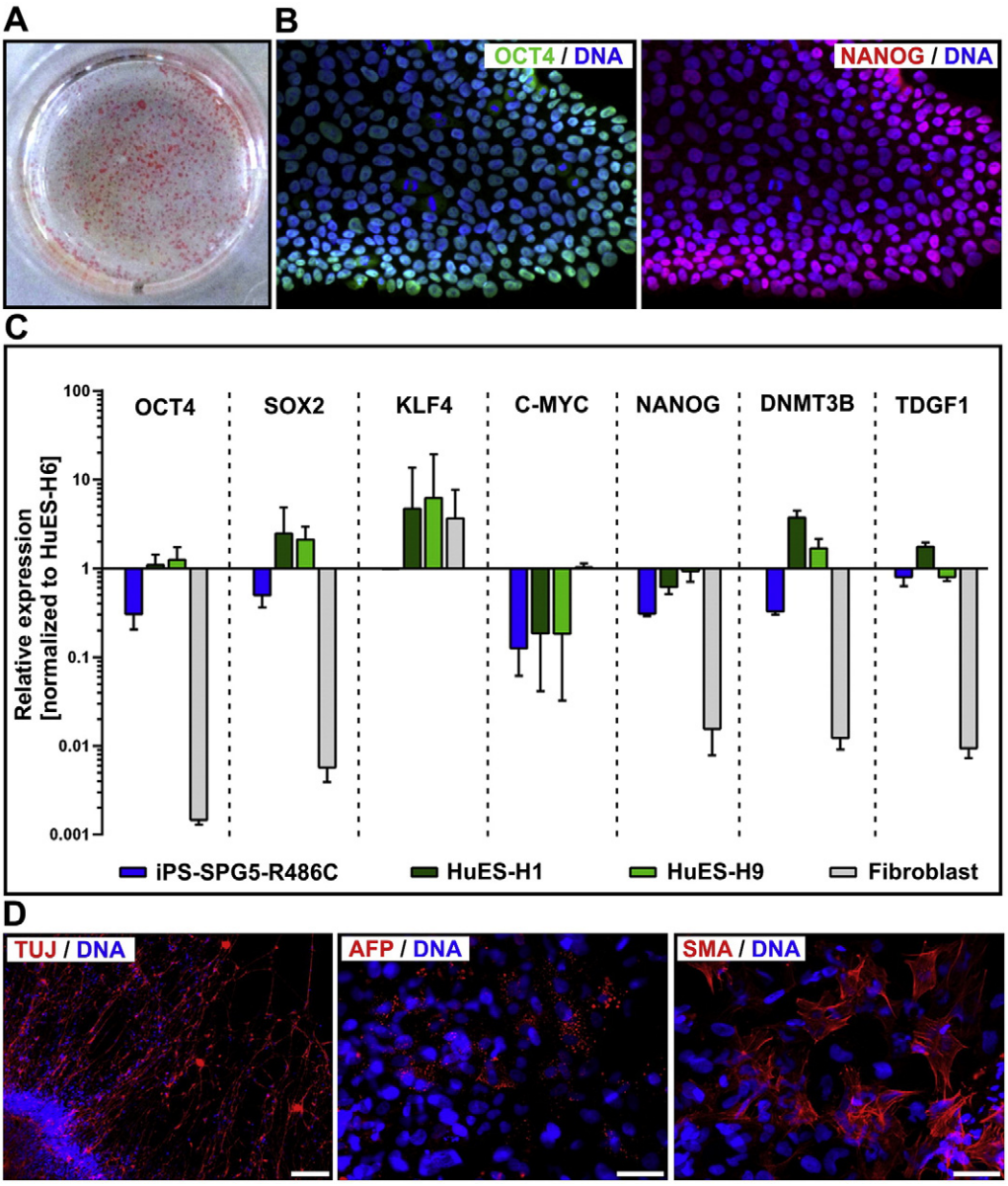

Fig. 2. Functional validation of iPS-SPG5-R486C. (A) Expression of alkaline phosphatase (ALP) (B) Immunocytochemical staining of OCT4 (green) and NANOG (red). Nuclei are counterstained with DAPI (blue). Scale bar = 50 μm (C) qRT-PCR with cDNA from iPS-SPG5-R486C, HuES-H1, HuES-H6, HuES-H9 and fibroblasts with the pluripotency genes OCT4, SOX2, KLF4, C-MYC, NANOG, DNMT3B and TDGF1 normalized to the housekeeping gene GAPDH and the hESC line HuES-H6. Expression levels of pluripotency markers are comparable to the gene expression in hESCs. (D) Immunocytochemical staining of *in vitro* differentiated iPSCs confirmed the ability to differentiate into cells of all three germ layers, namely ectoderm (β-III-tubulin (TUJ), scale = 100 μm), endoderm (α-fetoprotein (AFP), scale bar = 50 μm) and mesoderm (α-smooth-muscle-actin (SMA), scale bar = 50 μm). Nuclei are counterstained with DAPI (blue).

(E8) medium containing 100 μM NaB (Sigma-Aldrich). After approx. 21–28 days, iPSC colonies were picked manually and further expanded on Matrigel-coated 6-well dishes cultivated with E8 medium. After reaching confluency cells were split in a ratio of 1:6–1:12 with PBS/EDTA (0.02% EDTA in PBS) and further expanded. At passage 5–10, iPSCs were harvested for analysis or 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 manufacturer's guidelines. The SNP array was performed using the Affymetrix CytoScan HD technology (Affymetrix). For analysis, 2 μg of DNA of the original fibroblast line with the respective iPSC line were included. Raw data was analyzed using Affymetrix Chromsome Analysis Suite (ChAS) 2.0 software.

2.3. Sequencing of mutation site

Sanger sequencing of the mutation (c.1456C > T) of *CYP7B1* gene was carried out on 3130xl Genetic Analyzer (Applied Biosystems) using a primer set that flank the mutation site according to standard procedures and analyzed with the Staden 2.0.0b10 software (Staden Sourceforge).

2.4. Non-integration of transgenes

DNA was isolated using the DNeasy blood & tissue kit (Qiagen) according to manufacturer's guidelines. The three episomal plasmids (pCXLE-hUL, pCXLE-hSK and pCXLE-hOCT4) were used as positive

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

	Forward sequence	Reverse sequence
KLF4	CCACCTCGCCTTACACATGAAG	TAGCGTAAAAGGAGCAACATAG
L-MYC	GGCTGAGAAGAGGATGGCTAC	TTTGTTTGACAGGAGCGACAAT
OCT-3/4	CATTCAAACTGAGGTAAGGG	TAGCGTAAAAGGAGCAACATAG
SOX-2	TTCACATGTCCCAGCACTACCAG	TTTGTTTGACAGGAGCGACAAT

control. RT-PCR reactions were run with GoTaq G2 DNA Polymerase (Promega) according to manufacturer's instruction. PCR products were studied by gel electrophoresis on a 2% agarose gel with Midori Green. Gene Ruler DNA Ladder was used as size ladder. The plasmid specific primers are listed in Table 1.

2.5. Alkaline phosphatase staining

iPSCs were cultivated on 12-well plates until confluency of 60–80%. Cells were fixed in 4% paraformaldehyde (PFA) for 1 min and washed 3 times with PBS. Staining solution consist of 40 μl Naphthol AS-MX phosphate alkaline solution (Sigma-Aldrich) and 1 ml Fast Red (1 mg/ml, Sigma Aldrich). After 30 min ALP-positive colonies were stained dark red and solution was replaced by PBS.

2.6. Immunocytochemical staining

iPSCs were cultivated on 24-well plates on coverslips until confluency of 60–80%. Cells were fixed in 4% paraformaldehyde (PFA) for 15 min and washed 3 times with PBS. Permeabilization and blocking was achieved by incubation in blocking buffer (PBS, 1% FCS, 0.1% Triton X-100) for 45 min. Primary antibody (see Table 3) was applied for 1 h at room temperature (RT) followed by 3 times washing in PBS and subsequent incubation of secondary antibody (Alexa488 or Alexa568 diluted 1:300 (Life technologies)) in the dark at RT for 1 h. After 3 washing steps in PBS, DAPI (1:10,000) was used for nuclear counterstaining by incubation for 15 min at RT in the dark. Cells were embedded in ProLong Diamond Antifade Mountant. Images were captured with Axio Imager Z1 with ApoTome (Zeiss).

2.7. qRT-PCR of pluripotency marker

Total RNA was isolated from iPSCs using the High Pure RNA Isolation Kit (Roche) followed by reverse transcription into cDNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to manufacturer's guidelines. qPCR reactions were run with Light Cycler 480 SYBR Green I Master (Roche) and primers specific for pluripotency genes (see Table 2). CT-values were normalized using $2^{-\Delta\Delta Ct}$ with human embryonic stem cells (hESCs HuES-H6) as reference and GAPDH as housekeeping gene.

2.8. In vitro differentiation potential

iPSCs were detached and resuspended 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. On day 4, EBs were collected and plated on coverslips coated with 0.1% gelatine (Sigma-Aldrich). EB medium was exchanged every other day and cells were cultivated for additional 2–3 weeks. EBs were fixed for 15 min in 4% PFA for immunocytochemical analysis using AFP (α-fetoprotein), SMA (α-smooth muscle actin) and TUJ (β-III-tubulin) as antibodies for the three germ layers (see Table 3).

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
OCT-4	GGAAGGTATTCAGCCAAACG	CTCCAGGTTGCCTCTCACTC
SOX-2	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
In vitro differentiation	mouse anti-AFP	1:200	Sigma-Aldrich
	mouse anti-SMA	1:100	Dako
	Mouse anti-TUJ	1:1000	Sigma-Aldrich

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