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Lab resource: Stem Cell Line

Generation of optic atrophy 1 patient-derived induced pluripotent stem cells (iPS-OPA1-BEHR) for disease modeling of complex optic atrophy syndromes (Behr syndrome)

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ABSTRACT

Human skin fibroblasts were isolated from a 48-year-old patient carrying compound heterozygous mutations (c.610 + 364G>A and c.1311A>G) in *OPA1*, responsible for early onset optic atrophy complicated by ataxia and pyramidal signs (Behr syndrome; OMIM #210000). Fibroblasts were reprogrammed using episomal plasmids carrying *hOCT4*, *hSOX2*, *hKLF4*, *hL-MYC* and *hLIN28*. The generated transgene-free line iPS-OPA1-BEHR showed no additional genomic aberrations, maintained the disease-relevant mutations, expressed important pluripotency markers and was capable to differentiate into cells of all three germ layers *in vitro*. The generated iPS-OPA1-BEHR line might be a useful platform to study the pathomechanism of early onset complicated optic atrophy syndromes.

Resource table.

Name of stem cell line	iPS-OPA1-BEHR
Institution	German Center for Neurodegenerative Diseases (DZNE), Tuebingen, Germany
Person who created resource	Stefan Hauser, Stefanie Schuster, Yvonne Theurer
Contact person and email	Stefan Hauser, stefan.hauser@dzne.de
Date archived/stock date	February 2016
Origin	Human skin fibroblasts
Type of resource	Biological reagent: induced pluripotent stem cell (iPSCs); derived from a patient with compound heterozygous mutations (c.610 + 364G>A, c.1311A>G het) in *OPA1*
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-OPA1-BEHR 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

Resource details

In this study fibroblasts were isolated and reprogrammed from a 48-year-old female patient carrying compound heterozygous mutations (c.610 + 364G>A and c.1311A>G) in *OPA1*. The combination of a deep intronic mutation and a mutation in an intragenic modifier leads to a severe early-onset optic atrophy phenotype complicated by ataxia and pyramidal signs (Behr syndrome; OMIM #210000) (Bonifert et al., 2014) (Chao de la Barca et al., 2016). The iPSC line iPS-OPA1-BEHR was established by nucleofection of episomal plasmids carrying the sequence of *OCT4*, *SOX2*, *KLF4*, *L-MYC* and *LIN28* (Okita et al., 2011). To prove genomic integrity upon reprogramming, whole-genome single nucleotide polymorphism (SNP) genotyping of original fibroblasts and the generated iPSC line iPS-OPA1-BEHR was performed (Fig. 1A). Additionally both mutation-sites were resequenced (Fig. 1B) and genomic integration of episomal plasmids was excluded (Fig. 1C). Pluripotency of generated iPS-OPA1-BEHR was analysed *via* alkaline phosphatase staining (ALP) (Fig. 2A), staining of pluripotency markers OCT4, TRA-1-81 and SSEA-4 (Fig. 2B), comparative qRT-PCR analysis of pluripotency markers (OCT4, NANOG, KLF4, C-MYC, SOX2, REX1, DNMT3B and TDGF1) with human embryonic stem cells and fibroblasts (Fig. 2C) as well as by the capability to differentiate into cells of all three germ layers (ectoderm, mesoderm, endoderm) (Fig. 2D).

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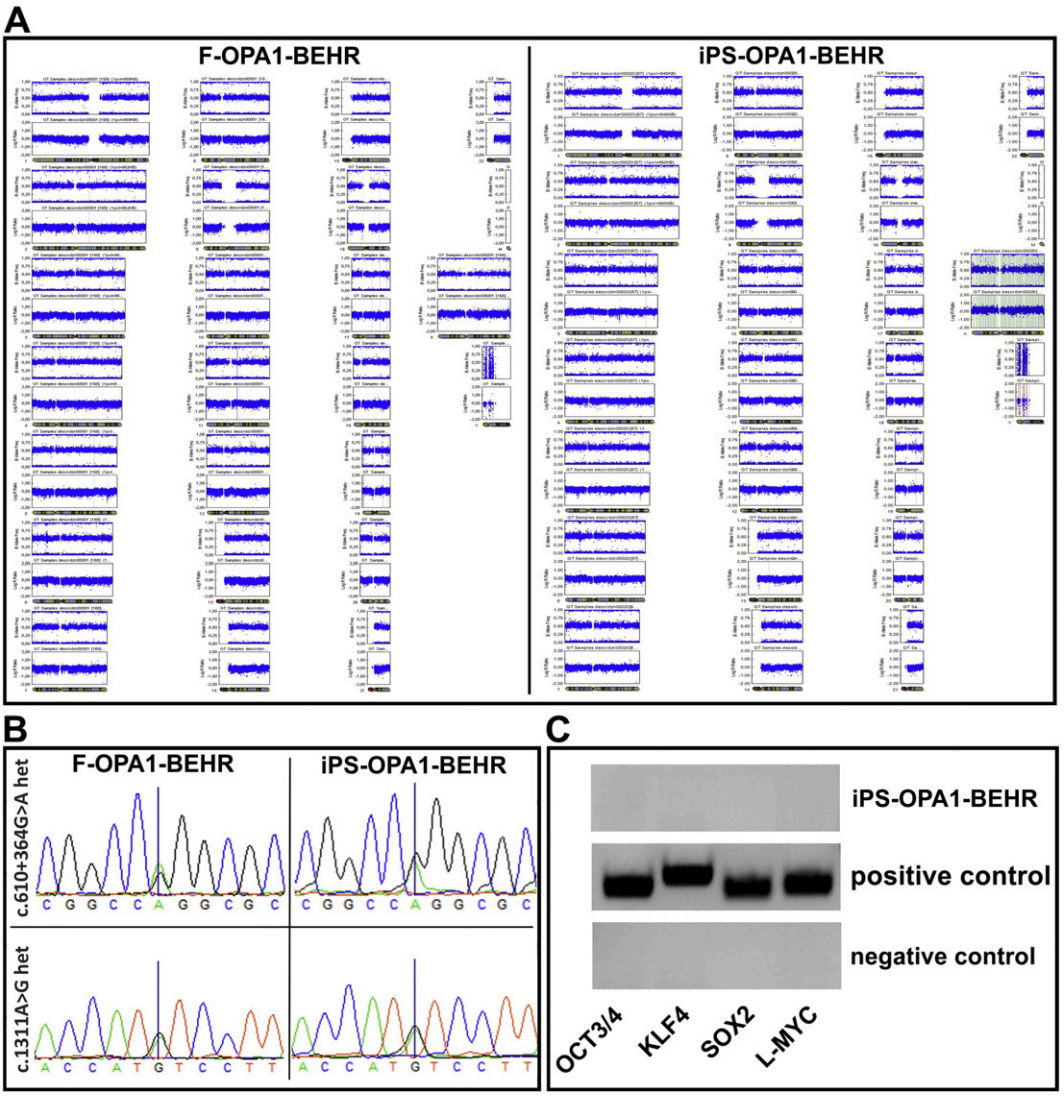

Fig. 1. Genomic validation of iPS-OPA1-BEHR. (A) Karyograms of iPSC line iPS-OPA1-BEHR with respective original fibroblast line. Every chromosome with the corresponding B allele frequency and log R ratio is displayed. (B) Sanger sequencing of patient-specific mutations. Sequences of iPS-OPA1-BEHR and F-OPA-BEHR show compound heterozygous mutations (c.610 + 364G>A and c.1311A>G) in *OPA1*. (C) Transgene-free state of iPSC line iPS-OPA1-BEHR proven *via* PCR with DNA from iPS-OPA1-BEHR and plasmid-specific primer for OCT3/4, SOX2, KLF4, and L-MYC. Plasmid samples as positive control, and ddH_2O as negative control.

Materials and methods

Reprogramming of fibroblast to iPSCs

Fibroblasts were obtained from a skin biopsy of a 48-year-old female patient carrying compound heterozygous mutations (c.610 + 364G>A and c.1311A>G; NM: 130837.1) in *OPA1*. The patient suffered from optic atrophy and visual loss since the age of 2 years. At 6 years of age progressive gait difficulties occurred due to cerebellar ataxia, spasticity and peripheral neuropathy as described in more detail in Bonifert et al. (2014). Skin tissue was dissected and directly plated in a 25 cm^2 tissue culture flask with 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. Fibroblasts were expanded by medium change every 2–3 days and reprogrammed by electroporation of 1×10^5 cells with a total of 1 μg per episomal plasmid (pCXLE-hUL, pCXLE-hSK and pCXLE-hOCT4) (Okita et al., 2011). After electroporation fibroblasts were cultivated for 1 day in fibroblast medium. On day 2 fibroblast growth factor-2 (FGF-2, 2 ng/ml, Peprotech) was added to the medium followed by medium change to Essential 8 (E8) supplemented with 100 μM NaB (Sigma Aldrich) on day 3. Medium change was performed every other day. iPSC colonies were manually picked approx. 3–4 weeks after electroporation and expanded on Matrigel-coated dishes in E8 medium. iPSCs were split by adding PBS/EDTA (0.02% EDTA in PBS) and replated in a ratio of 1:6–1:12. Around passage 5–10 iPSCs were genomically and functionally analysed and frozen in E8 medium supplemented with 40% KOSR (Life Technologies), 10% DMSO (Sigma-Aldrich), and 1 μM Y-27632 (Abcam Biochemicals).

SNP array analysis

DNA of iPSCs was isolated with DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's instructions. Whole-genome SNP genotyping was conducted using the Infinium OmniExpressExome-8 BeadChip (Illumina). Data analysis was performed with Illumina BeadStudio.

Sequencing of mutation site

DNA from the patient's fibroblasts and iPSCs was extracted using the DNeasy blood & tissue kit (Qiagen) according to manufacturer's guidelines. Subsequently, Sanger sequencing of both mutations

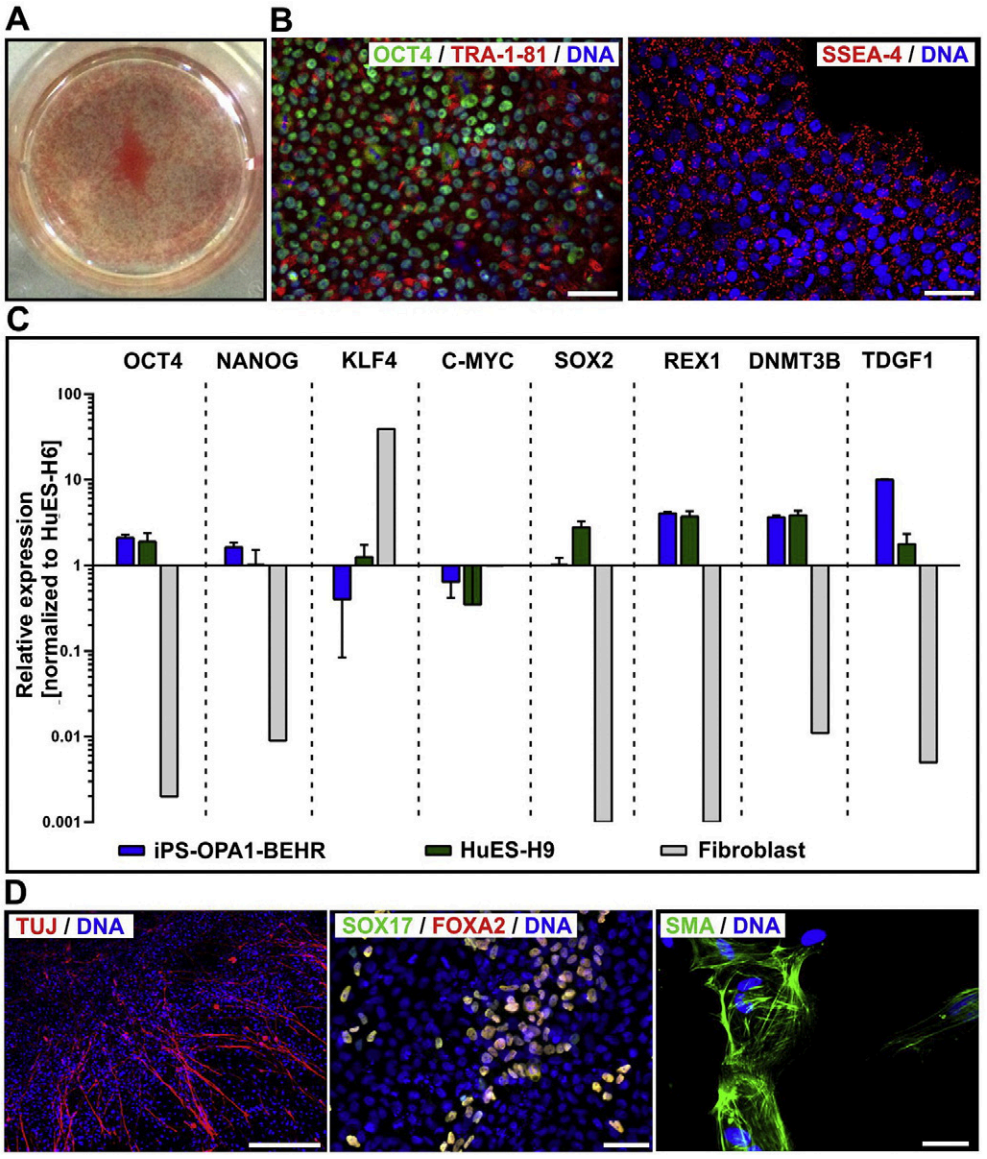

Fig. 2. Functional validation of iPS-OPA1-BEHR. (A) Dark-red staining of alkaline phosphatase assay. (B) Pluripotency marker (OCT4, TRA-1-81 and SSEA-4) identified by immunocytochemical staining of iPS-OPA1-BEHR. Nuclei are counterstained with DAPI (blue). Scale bar = 50 μm. (C) qRT-PCR of iPS-OPA1-BEHR, fibroblasts and the human embryonic stem cell lines HuES-H6 and HuES-H9 with the pluripotency genes OCT4, NANOG, KLF4, C-MYC, SOX2, REX1, DNMT3B and TDGF1. Data as triplicates and normalized to the housekeeping gene GAPDH and the hESCs HuES-H6. (D) iPS-OPA1-BEHR line is able to differentiate into cells of all three germ layers demonstrated by immunocytochemical staining (ectoderm (β-III-tubulin (TUJ), scale = 100 μm), endoderm (SOX17 and FOXA2, scale bar = 50 μm) and mesoderm (α-smooth-muscle-actin (SMA), scale bar = 50 μm)). Nuclei are counterstained with DAPI (blue).

(c.610 + 364G>A and c.1311A>G) in OPA1 was carried out using specific primer sets flanking the mutation sites according to standard procedures by using 3130xl Genetic Analyzer (Applied Biosystems) and Staden 2.0.0b10 software (Staden Sourceforge) for visualisation.

Non-integration of transgenes

DNA was isolated as described before. Using the GoTaq G2 DNA Polymerase (Promega) according to manufacturer's instruction as well as plasmid-specific primers (see Table 1) genomic integration of the episomal plasmids (pCXLE-hUL, pCXLE-hSK and pCXLE-hOCT4) was excluded. *Via* gel electrophoresis on a 2% agarose gel with Midori Green and Gene Ruler DNA Ladder PCR products were analysed.

Alkaline phosphatase staining

IPSCs were fixed in 4% paraformaldehyde (PFA) for 1 min and washed 3 times with PBS after reaching a confluency of approx. 60–80%. Staining solution (40 μl Naphthol AS-MX phosphate alkaline

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

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

solution (Sigma-Aldrich) and 1 ml Fast Red (1 mg/ml, Sigma Aldrich)) was applied for 30 min leading to dark red staining of ALP-positive iPSC colonies.

Immunocytochemical staining

iPSCs as well as differentiated cells were fixed in 4% PFA for 15 min and washed 3 times with PBS. Subsequently, blocking solution (PBS supplemented with 1% FBS and 0.1% Triton X-100) was added for 45 min and samples were incubated with primary antibody (see Table 3) for 1 h at room temperature (RT). After 3 times washing in PBS secondary antibody (Alexa488 or Alexa568 diluted 1:300 (Life Technologies)) was applied and incubated for 1 h at RT in the dark. Nuclear counterstaining was achieved by adding DAPI (1:10,000) for 15 min at RT. After embedding cells in ProLong Diamond Antifade Mountant (Life Technologies) images were taken with Axio Imager Z1 with ApoTome (Zeiss).

qRT-PCR of pluripotency marker

cDNA generated by using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) as well as prior isolated total RNA (High Pure RNA Isolation Kit (Roche)) according to manufacturer's guidelines was used for qRT-PCR analysis. qRT-PCR was performed as triplicates with Light Cycler 480 SYBR Green I Master (Roche) and primers specific for pluripotency genes (see Table 2). Normalization of C_T-values was achieved by using the $2^{-\Delta\Delta Ct}$ method with the human embryonic stem cell line HuES-H6 as reference and GAPDH as housekeeping gene.

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

	Antibody	Dilution	Manufacturer
Pluripotency	Goat anti-OCT4	1:100	Santa-Cruz
	Mouse anti-SSEA4	1:500	Abcam
	Mouse anti-TRA-1-81	1:500	Merck-Millipore
In vitro differentiation	Rabbit anti-FOXA2	1:300	Merck
	Mouse anti-SMA	1:100	Dako
	Goat anti-SOX17	1:250	R&D systems
	Mouse anti-TUJ	1:1000	Sigma-Aldrich

In vitro differentiation potential

For proving the ectodermal and mesodermal differentiation potential iPSCs were detached and resuspended in Embryoid Body (EB) medium (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 and plated on 0.1% gelatine-covered wells (Sigma-Aldrich) at day 4. EB medium was exchanged every other day and cells were cultivated for additional 2–3 weeks. Endodermal differentiation of iPSCs was achieved by addition of endodermal differentiation medium (RPMI1640 (Merck) supplemented with 1× B27 (Life Technologies), 0.2% FBS, 2 μM CHIR-99021 (Abcam) and 50 ng/ml Activin A (Peprotech)) for 5 days. CHIR-99021 was removed from day 2 on and medium was changed every day.

Acknowledgements

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