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Lab Resource: Stem Cell Line

Induced pluripotent stem cells (iPSCs) derived from cerebrotendinous xanthomatosis (CTX) patient's fibroblasts carrying a R395S mutation

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ABSTRACT

Induced pluripotent stem cells (iPSCs) were generated from dermal fibroblasts from a 60-year-old cerebrotendinous xanthomatosis (CTX) patient, carrying a homozygous mutation c. [1183C>A]; p. R395S in *CYP27A1*. Episomal plasmids encoding the pluripotency genes *OCT4, SOX2, KLF4, L-MYC* and *LIN28* were introduced *via* electroporation. The generated line iPS-CTX-R395S has no sign of plasmid integration or chromosomal aberration and retained the mutation site in *CYP27A1*. Furthermore, iPSCs express pluripotency markers and are able to differentiate in all germ layers *in vitro*. The generated line may be a useful tool for disease modelling of CTX.

Resource table.

Name of stem cell line	iPS-CTX-R395S
Institution	German Center for Neurodegenerative Diseases (DZNE), Tuebingen, Germany
Person who created resource	Philip Höflinger, Stefan Hauser, Yvonne Theurer, Stefanie Weißenberger
Contact person and email	Stefan Hauser, stefan.hauser@dzne.de
Date archived/stock date	January 2016
Origin	Human dermal fibroblasts
Type of resource	Biological reagent: induced pluripotent stem cell (iPSCs) derived from a cerebrotendinous xanthomatosis (CTX) patient carrying a homozygous R395S mutation
Sub-type	Induced pluripotent stem cells (iPSCs)
Key transcription factors	*hOCT4, hSOX2, hKLF4, hL-MYC, hLIN28* (Addgene plasmids 27076, 27078; and 27080; Okita et al., 2011)
Authentication	Identity and purity of iPS-CTX-R395S line confirmed by analysis of plasmid integration, mutation resequencing, SNP array analysis (Fig. 1), expression of 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

Cerebrotendinous xanthomatosis (CTX) is an autosomal recessive lipid storage disease. Mutations in *CYP27A1* lead to defects in the mitochondrial enzyme sterol 27-hydroxylase involved in the synthesis of bile acids from cholesterol. A block in this pathway due to loss-of-function mutations in *CYP27A1* leads to high amounts of abnormal lipids like cholestanol in serum and cerebrospinal fluid (CSF) and their subsequent deposition in the ocular lens resulting in juvenile cataract and in xanthomas, predominately in tendons. Additional symptoms can include chronic diarrhea due to lack of bile acids, atherosclerosis, osteoporosis as well as progressive neurological dysfunctions such as ataxia, dystonia, peripheral neuropathy and dementia (Nie et al., 2014).

In this study, we reprogrammed dermal fibroblasts from a 60-year-old female CTX patient, carrying a homozygous c. [1183C>A]; p. R395S mutation in *CYP27A1*, to induced pluripotent stem cells (iPSCs). Functional relevance of the mutation was confirmed by massively increased levels of cholestanol (3.41 mg/dl; normal: 0.36 ± 0.09). Episomal plasmids encoding the pluripotency genes *OCT4*, *SOX2*, *KLF4*, *L-MYC* and *LIN28* were introduced *via* electroporation (Okita et al., 2011). The genetic integrity of iPS-CTX-R395S was confirmed by comparative SNP analysis with the parental fibroblasts (Fig. 1A), resequencing of the mutation site (Fig. 1B), and the exclusion of integrated reprogramming plasmids by PCR (Fig. 1C). Gene expression of the stem cell markers alkaline phosphatase (ALP) (Fig. 2A), OCT4, and NANOG (Fig. 2B) was shown by enzymatic activity or immunocytochemistry. Expression of OCT4, SOX2, KLF4, c-MYC, NANOG, DNMT3B and TDGF1 was verified by qRT-PCR in comparison to the human embryonic stem cell lines HuES-H6 and HuES-H9 and fibroblasts (Fig.

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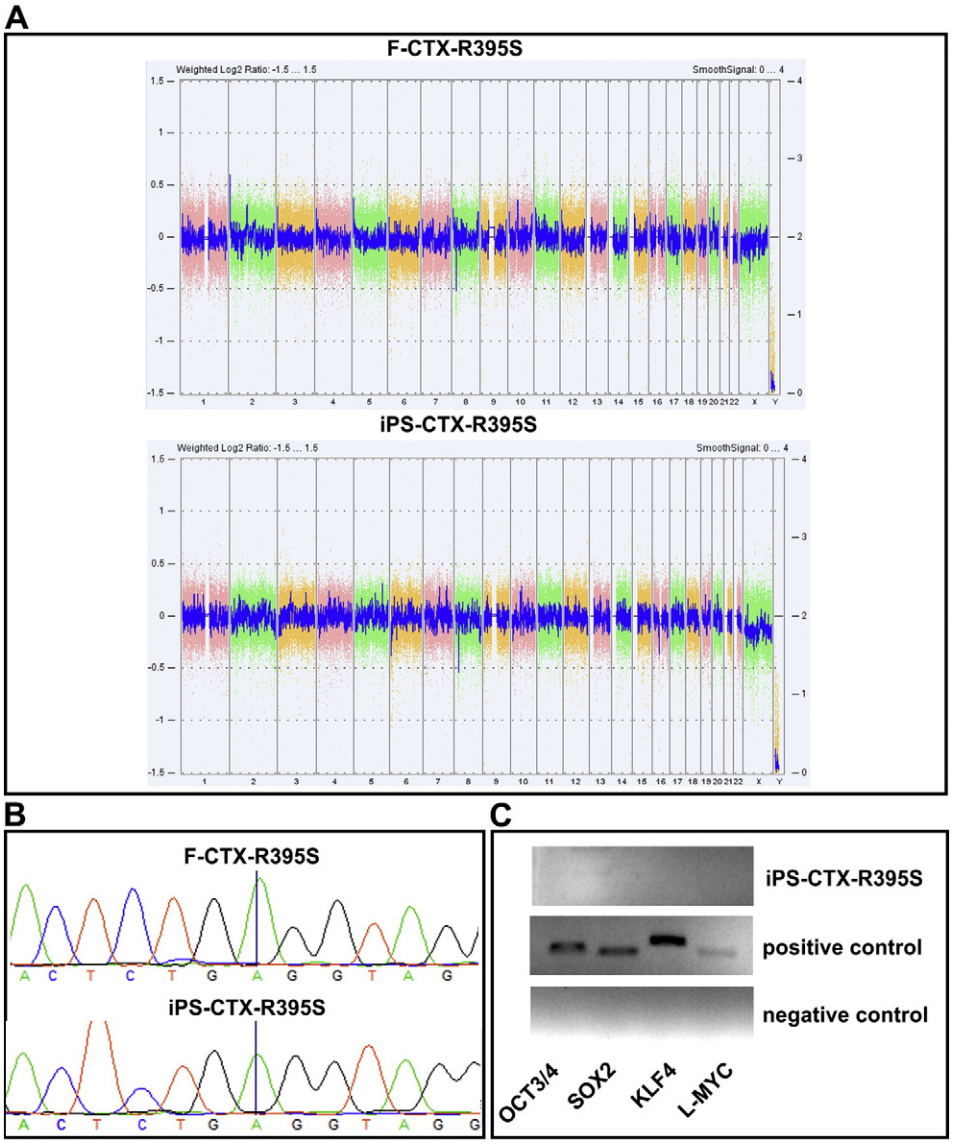

Fig. 1. Genomic characterization of iPS-CTX-R395S. (A) Comparison of SNP array analysis of the fibroblast line F-CTX-R395S and the iPSC line iPS-CTX-R395S shows chromosomal integrity after reprogramming. Data is represented as the weighted log2 ratio of the copy number on the left Y-axis (blue line) and the chromosome number on the X-axis. (B) Electropherograms confirm the patient-specific homozygous point mutation c.1183C>A. (C) Absence of integrated plasmids identified by PCR with DNA from iPS-CTX-R395S, the reprogramming plasmids as positive control, and water as negative control using plasmid-specific primers for OCT3/4, SOX2, KLF4, and L-MYC.

2C). Spontaneous differentiation was performed showing the potential of the generated line to differentiate into all three germ layers. Ectodermal lineage was proven by neuronal cells expressing β-III-Tubulin, endodermal lineage by cells expressing α-Fetoprotein, and mesodermal lineage by muscle cells expressing smooth muscle actin (SMA) (Fig. 2D).

Materials and methods

Reprogramming of fibroblast to iPSCs

The patient's skin biopsy was dissected and subsequently cultured in Dulbecco's modified eagle's medium (DMEM) high glucose (Life Technologies) with 10% fetal bovine serum (FBS, Life Technologies) for around 10 days at 37 °C and 5% CO_2 with medium changes every 2–3 days until fibroblasts grew out of the biopsy. Reprogramming was achieved by electroporation of 1×10^5 fibroblasts using a Nucleofector 2D (Lonza) and 1 μg of each plasmid pCXLE-hOCT3/4, pCXLE-hSK, and pCXLE-hUL (Okita et al., 2011). After 1 day in DMEM + 10% FBS, 2 μg/l fibroblast growth factor 2 (FGF2, Peprotech) was added to the medium. From day 3 on cells were cultured in essential 8 (E8) medium consisting of DMEM/F12 (Life Technologies), 64 mg/l L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma-Aldrich), 1× ITS-Supplement (Life Technologies), 10 μg/l FGF2, 2 μg/l TGF-β-1 (Peprotech), 100 μg/l heparin (Sigma-Aldrich), and 100 μM Na-butyrate (NaB) (Sigma-Aldrich) with medium change every other day. Three to four weeks after reprogramming iPSC colonies were picked manually and transferred to Matrigel (Corning)-coated 6-well plates for further expansion. Cells were split with 0.02% EDTA in PBS at ratios between 1:6 and 1:12 until reaching passage 5–10 when further analysis was carried out.

SNP array analysis

DNA was isolated from iPSCs and the original fibroblast line using the DNeasy blood & tissue kit (Qiagen) according to manufacturer's guidelines. 2 μg of each DNA was analyzed using the Affymetrix CytoScan HD technology (Affymetrix) and raw data was visualized with the Affymetrix Chromosome Analysis Suite (ChAS) 2.0 software.

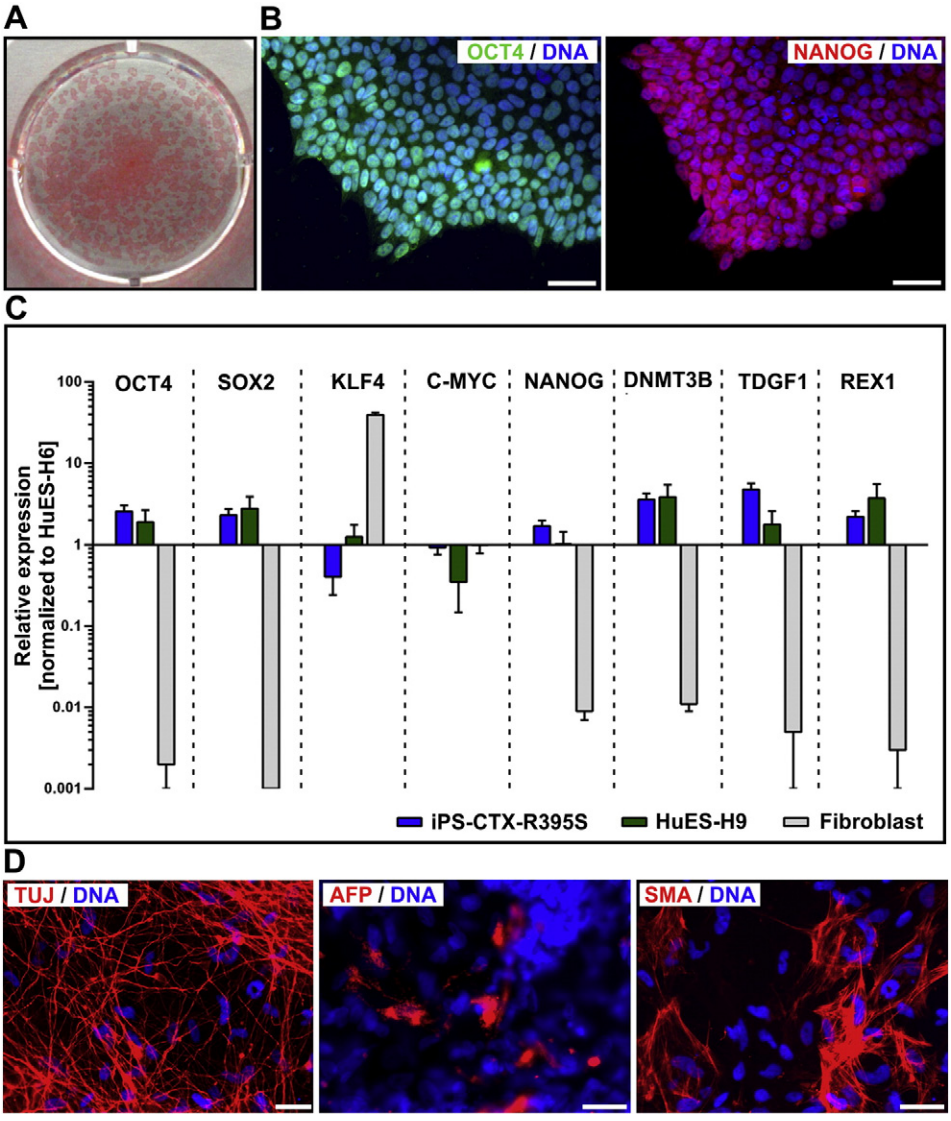

Fig. 2. Functional characterization of iPS-CTX-R395S by (A) alkaline phosphatase staining (ALP). (B) Immunocytochemical staining of OCT4 (green) and NANOG (red). Nuclei are counterstained with DAPI (blue). Scale bar = 50 μm. (C) qRT-PCR-based expression analysis of stem cell markers OCT4, SOX2, KLF4, c-MYC, NANOG, DNMT3B, TDGF1, and REX1. Expression is normalized to the reference gene GAPDH and in relation to the human embryonic stem cell line HuES-H6. (D) Immunocytochemical staining of *in vitro* differentiated iPSCs expressing markers of all germ layers, β-III-Tubulin (TUJ, ectoderm), α-Fetoprotein (AFP, endoderm), and SMA (smooth muscle actin, mesoderm). Nuclei are counterstained with DAPI (blue). Scale bar is 100 μm for TUJ and 50 μm for AFP and SMA.

Sequencing of mutation site

The mutation site [c.1183C>A] in the *CYP27A1* gene was Sanger sequenced on a 3130xl Genetic Analyzer (Applied Biosystems). A primer pair flanking the mutation site was used for PCR and the Amplicon was analyzed with the Staden 2.0.0b10 software (Staden Sourceforge).

Non-integration of transgenes

DNA was isolated from iPS-CTX-R395S using the DNeasy blood & tissue kit (Qiagen) according to manufacturer's guidelines. PCR reactions were conducted with the GoTaq G2 DNA Polymerase (Promega) according to manufacturer's instruction. As a positive control, the three plasmids applicated for reprogramming were used (primer sequences are listed in Table 1). PCR products were analyzed by gel electrophoresis on a 2% agarose gel using Midori Green as a dye. As a size ladder Gene Ruler DNA ladder was used.

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

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
	Rabbit anti-NANOG	1:50	Stemgent
In vitro differentiation	Mouse anti-AFP	1:200	Sigma-Aldrich
	Mouse anti-SMA	1:100	Dako
	Mouse anti-TUJ	1:1000	Sigma-Aldrich

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 phosphate buffered saline (PBS). Cells were stained for 30 min in 40 μl Naphthol AS-MX phosphate alkaline solution (Sigma-Aldrich) and 1 ml Fast Red (1 mg/ml, Sigma Aldrich). ALP-positive colonies were stained dark red and the staining solution was replaced by PBS.

Immunocytochemical staining

iPSCs were cultured on coverslips in 24-well plates until a confluency of 60–80%. For staining, cells were fixed in 4% paraformaldehyde (PFA) for 15 min followed by three washing steps with PBS. Cells were incubated in PBS, 1% FCS, and 0.1% Triton X-100 for 45 min for blocking and permeabilization. The primary antibodies (Table 3) were applied for 1 h at RT followed by three times washing in PBS, incubation of secondary antibodies Alexa Fluor 488 or Alexa Fluor 568 (1:300) for 1 h at RT in the dark and again three washing steps in PBS. DAPI (1:5000) was used for nuclear counterstaining. Finally, cells were embedded in mounting medium (Dako) and imaged with Axio Imager Z1 with ApoTome (Zeiss).

qRT-PCR of pluripotency marker

Total RNA was isolated from cell samples using the high Pure RNA isolation Kit (Roche) following cDNA synthesis by reverse transcription using the Transcriptor High Fidelity cDNA Synthesis Kit according to the manufacturer's guidelines. For further analysis, cDNA was diluted 1:10 and the qPCR reactions were prepared with LightCycler 480 SYBR Green I Master Kit (Roche) and executed on a LightCycler 480 (Roche). The experiments were performed as triplicates and analyzed using the ΔΔCT method with GAPDH as a reference gene and hESC line HuES-H6 for normalization (Table 2) .

In vitro differentiation potential

The differentiation potential of iPS-CTX-R395S was investigated by embryoid body (EB) formation. Therefore, iPSCs were detached from the cell culture plate and cultured in Aggrewell800 plates (Stemcell Technologies) in EB medium (80% DMEM/F12 (Life Technologies), 20% KOSR, 1× NEAA, 1× Pen/Strep, 2 mM L-Glutamine and 0.1 mM 2-Mercaptoethanol). After two days, EBs were transferred to gelatine (0.1% in sterile ddH_2O) coated 24-well plates and cultivated in EB medium. Medium was changed every other day for two weeks. Cells were fixed in 4% PFA for 15 min and stained for germ layer specific markers, namely β-III-Tubulin (ectoderm), α-Fetoprotein (endoderm), and smooth muscle actin (mesoderm) (see Table 3).

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