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Lab resource: Stem Cell Line

Generation of a human induced pluripotent stem cell (iPSC) line from a 51-year-old female with attention-deficit/hyperactivity disorder (ADHD) carrying a duplication of *SLC2A3*

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ABSTRACT

Fibroblasts were isolated from a skin biopsy of a clinically diagnosed 51-year-old female attention-deficit/hyperactivity disorder (ADHD) patient carrying a duplication of *SLC2A3*, a gene encoding neuronal glucose transporter-3 (GLUT3). Patient fibroblasts were infected with Sendai virus, a single-stranded RNA virus, to generate transgene-free human induced pluripotent stem cells (iPSCs). *SLC2A3*-D2-iPSCs showed expression of pluripotency-associated markers, were able to differentiate into cells of the three germ layers *in vitro* and had a normal female karyotype. This *in vitro* cellular model can be used to study the role of risk genes in the pathogenesis of ADHD, in a patient-specific manner.

Resource table

Unique stem cell line identifier	UKWi001-A
Alternative name(s) of stem cell line	*SLC2A3*-D2-iPSC
Institution	Division of Molecular Psychiatry, Center of Mental Health, University of Würzburg
Contact information of distributor	Klaus-Peter Lesch, kplesch@mail.uni-wuerzburg.de
Type of cell line	iPSC
Origin	human
Additional origin info	Age: 51 Sex: female
Cell source	Dermal fibroblasts
Clonality	Clonal
Method of reprogramming	Sendai virus
Genetic modification	No
Type of modification	N/A
Associated disease	Attention-deficit/hyperactivity disorder (ADHD)
Gene/locus	Duplication of *SLC2A3*, 12p13.31
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	June, 2017
Cell line repository/bank	N/A
Ethical approval	Ethics Committee, Medical Faculty, University of Würzburg, 96/11

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Resource utility

SLC2A3-D2-iPSCs represent a unique tool to clarify the role of *SLC2A3* as a risk gene for ADHD as well as its effect on the serotonin system by generating iPSC-derived serotonin-specific neurons.

Resource details

The pathogenesis of neurodevelopmental and psychiatric diseases such as ADHD is thought to underlie a complex interplay of multiple genes and environmental factors causing abnormalities in brain development. Among these abnormalities, deficiencies in transmitters such as serotonin may play a role in these processes. ADHD is characterized by inattention, hyperactivity and increased impulsivity. One of the genes associated with an increased risk for ADHD is *SLC2A3*, which encodes for glucose transporter-3 (GLUT3) (Lesch et al., 2011). As the predominant neuronal glucose transporter, GLUT3 is critically involved in cerebral glucose metabolism, providing energy for neuronal activity, which, in turn, moderates the brain's excitatory–inhibitory balance impacting both neurodevelopmental processes and activity-dependent neural plasticity (Merker et al., 2017). Therefore, GLUT3 dysfunction may contribute to the pathophysiology of neuropsychiatric disorders. In this study, adult dermal fibroblasts were isolated from a 51-year-old female ADHD patient carrying a duplication of *SLC2A3* (Fig. 1A). Patient fibroblasts were reprogrammed into induced pluripotent stem cells (iPSCs) using Sendai virus, transiently overexpressing OCT-3/4, SOX2, cMYC and KLF4. A few days after infection, a change in cell morphology was observed, which persisted until stem cell-like colonies formed on day 9 (Table 1). iPSC-like colonies were isolated manually 47 days post reprogramming and expanded under feeder-free conditions using StemMACS™ iPS-Brew XF. *SLC2A3*-D2-iPS cell colonies displayed typical ES-like morphology of compact, dense, roundly shaped colonies with sharp edges and typical ES-like growth behaviour (Fig. 1B). Standard G-banding revealed a normal female karyotype without numerical or structural chromosome abnormalities of *SLC2A3*-D2-iPSCs (46, XX) (Fig. 1C). Moreover, flow cytometric analysis confirmed the pluripotent identity of *SLC2A3*-D2-iPSCs through homogeneous expression of pluripotency specific markers TRA-1-60 and SSEA-4 (double positive cells: 97.5%) (Fig. 1D, Table 2). Comparative genomic hybridization (Array-CGH) confirmed *SLC2A3*-D2-iPSCs carrying a duplication of the *SLC2A3* gene locus with a size of 127,229 kb (chr12: 8,000,006-8,127,235; hg19) (Fig. 1E). The duplicated chromosomal region comprises the complete gene locus of *SLC2A3* and the initial exons of *SLC2A14*, a paralog of *SLC2A3*. Additionally, immunofluorescence (IF) analyses proved the expression of the surface markers TRA-1-60 and SSEA-4 and the transcription factor OCT-3/4 (Fig. 1F, Table 2). The absence of Sendai virus specific transcripts was ratified by reverse transcriptase (RT)-PCR after 20 passages (Fig. 1G). Furthermore, fibroblasts and *SLC2A3*-D2-iPSCs were confirmed as mycoplasma-free before and after pluripotency induction (Fig. 1H). Germ layer differentiation demonstrated the default differentiation capacity of *SLC2A3*-D2-iPSCs into embryoid bodies (EBs) when cultured in serum containing medium (Fig. 1I). EBs were cultured for 7 days in suspension and terminally differentiated in adherent culture conditions for up to 3 weeks. *SLC2A3*-D2-iPSCs differentiated towards mesodermal, endodermal and ectodermal layer specific cells as observed by positive IF staining against alpha-smooth muscle actin (SMA), alpha-1- fetoprotein (AFP) and beta-Tubulin III (ßTubIII), respectively (Fig. 1J, Table 2). Short tandem repeat (STR) analysis showed that parental fibroblasts and newly created *SLC2A3*-D2-iPSCs shared alleles with 100% match (submitted in archive with journal). Taken together, we successfully generated iPSCs that can be used to study the effects of a *SLC2A3* duplication on neurodevelopmental processes and therefore to unveil the involvement of this gene in the cellular pathophysiology of ADHD.

Materials and methods

Skin biopsy preparation

Human dermal fibroblasts were isolated from an ADHD patient carrying a *SLC2A3* duplication and cultured in fibroblast growth medium (Dulbecco's modified eagle's medium (DMEM; PAN-Biotech), 10% fetal bovine serum (FBS; Gibco), 1 μg/ml Gentamicin (Thermo Fisher)).

Reprogramming of patient fibroblasts into iPSCs

Fibroblasts of passage 3 were reprogrammed using CytoTune-iPS Reprogramming Kit 2.0 (Thermo Fisher). 75,000 fibroblasts per well of a 12-well plate were transduced with Sendai virus for 24 h. After 7 days cells were seeded on irradiated mouse embryonic fibroblasts (MEF; 400,000 MEF/6well) (Amsbio) in reprogramming medium (KnockOut™ DMEM/F12 (Gibco), 20% KnockOut™ Serum Replacement (Gibco), 2 mM L-glutamine (Gibco), 1% MEM non-essential amino acid solution (100×) (NEAA; PAN-Biotech), 100 μM β-mercaptoethanol (Gibco), 10 ng/ml human fibroblast growth factor 2 (FGF2; Gibco) and 200 μM ascorbic acid (Sigma-Aldrich)). Colonies with hESC-like appearance were manually transferred on day 47 post infection to Matrigel-coated 6-well plates in StemMACS™ iPS-Brew XF (Miltenyi Biotec) supplemented with 10 μM ROCK inhibitor (RI; Miltenyi Biotech).

Karyotype analysis

Karyotypes were verified by GTG-banding analysis. Cells were treated with 10 μg/ml Colcemid™ Solution and processed with standard methods. 10 metaphases per sample were examined with a resolution of 450–500 bands using Axioskop (Zeiss).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from 3×10^6 iPSCs, infected and non-infected HEK cells as controls (RNeasy mini Kit; Qiagen). 500 ng RNA was reverse transcribed with QuantiTect Reverse Transcription Kit (Qiagen). Sendai virus specific primers were used to assess the presence of remaining Sendai virus (Table2).

Flow cytometry

iPSCs were incubated with Viobility 405/452 Fixable Dye (Miltenyi Biotec) and directly conjugated antibodies (Table 2) for 15 min at 4 °C. Dead cells labeled by Viobility 405/452 Fixable Dye were excluded from the analysis. Data was acquired on a BD FACSCanto II flow cytometer (BD Biosciences).

Mycoplasma contamination detection

The absence of mycoplasma contamination was confirmed using LookOut® Mycoplasma PCR Detection Kit (Sigma-Aldrich).

Germ layer differentiation

iPSCs were seeded on ultra-low attachment plates (Corning) in differentiation medium (DMEM, 10% FBS, 1% NEAA, 100 μM β-mercaptoethanol) with 10 μM RI. After 7 days in suspension, EBs were plated on 0.1% gelatine (PAN Biotech)-coated plates for undirected differentiation and specific germ layer markers were tested by immunofluorescence staining 3 weeks later.

Immunofluorescence analysis

Cells were fixed with 4% paraformaldehyde (Roth), blocked (5% FBS in PBS) and permeabilized with 0.2% Triton X-100 (intracellular

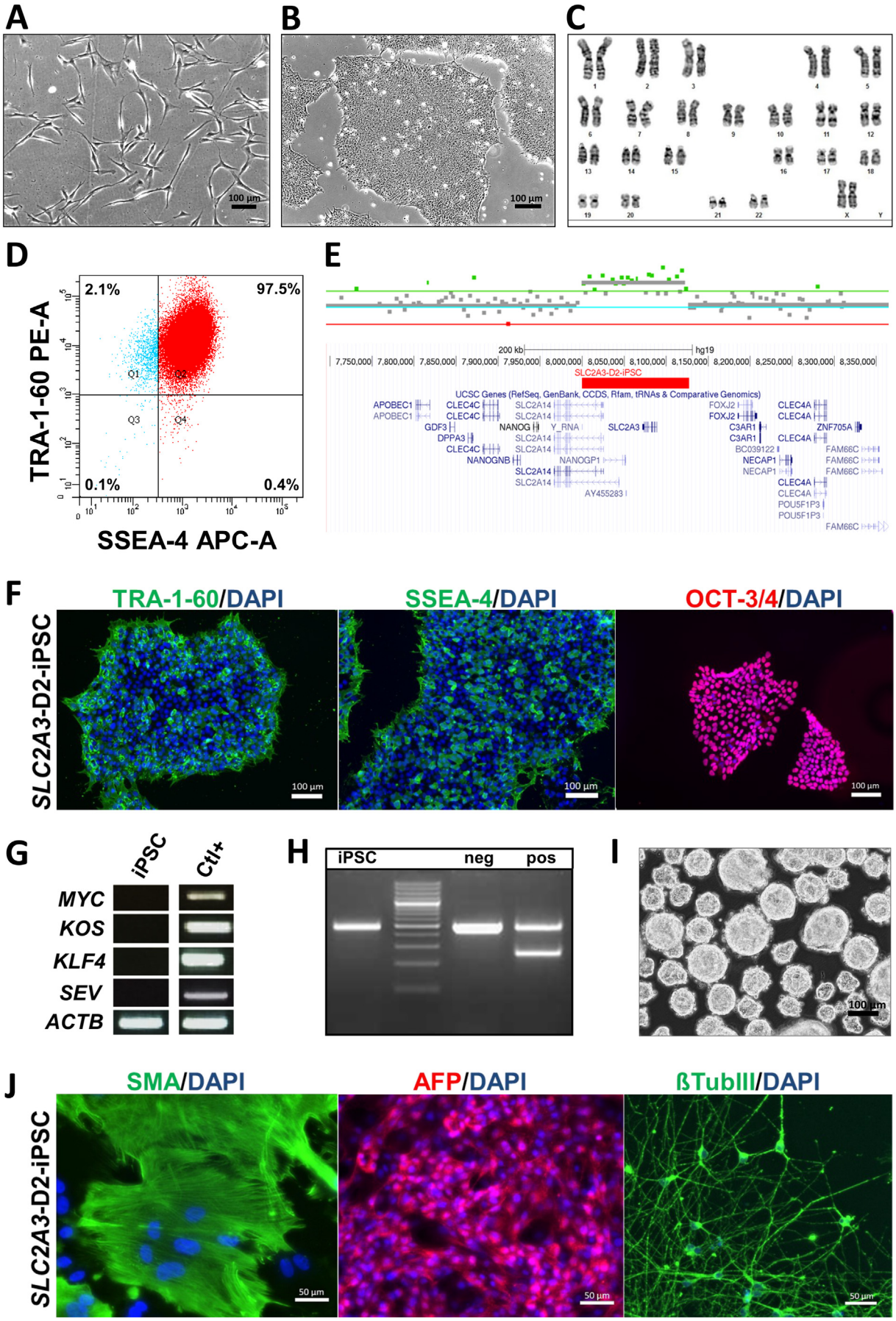
A
B
C
100 µm
100 µm
D
TRA-1-60 PE-A
SSEA-4 APC-A
2.1%
97.5%
0.1%
0.4%
Q1
Q2
Q3
Q4
E
200 kb
hg19
SLC2A3-D2-iPSC
UCSC Genes (RefSeq, GenBank, CCDS, Rfam, tRNAs & Comparative Genomics)
F
TRA-1-60/DAPI
SSEA-4/DAPI
OCT-3/4/DAPI
SLC2A3-D2-iPSC
100 µm
G
iPSC
Ctl+
MYC
KOS
KLF4
SEV
ACTB
H
iPSC
neg
pos
I
100 µm
J
SMA/DAPI
AFP/DAPI
ßTubIII/DAPI
SLC2A3-D2-iPSC
50 µm

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Light microscopy	hESC-like morphology (compact, dense, roundly shaped colonies with sharp edges)	Fig. 1 panel B
Genotype	Karyotype (G-banding)	46, XX; Resolution 450–500	Fig. 1 panel C
Phenotype	Quantitative analysis (Flow cytometry)	TRA-1-60/SSEA-4 double positive cells: 97.5%	Fig. 1 panel D
	Qualitative analysis (Immunofluorescence staining)	Expression of pluripotency-associated markers TRA-1–60, SSEA-4 and OCT-3/4: positive	Fig. 1 panel F
Mutation analysis	Array-CGH	Duplication of *SLC2A3*	Fig. 1 panel E
Identity	STR analysis	STR profile consists of 21 specific markers Cell line authentication of fibroblast cell line and iPSC line matched	Submitted in archive with journal
Microbiology and virology	Mycoplasma	LookOut® Mycoplasma PCR Detection Kit: negative	Fig. 1 panel H
Differentiation potential	Embryoid body formation and undirected differentiation	Expression of smooth muscle actin, α-feto protein and beta-Tubulin III: positive	Fig. 1 panels I, J
Donor screening	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Mouse anti-OCT-3/4	1:50	Santa Cruz, Cat# sc5279, RRID:AB_628051
	Mouse anti TRA-1-60	1:50	Santa Cruz, Cat# sc21705, RRID:AB_628385
	Mouse anti-SSEA-4	1:200	Thermo Fisher, Cat# MA1-021, RRID:AB_2536687
Differentiation markers	Mouse anti-betaTubIII	1:1000	Promega, Cat# G7121, RRID:AB_430874
	Rabbit anti-AFP	1:400	Dako, Cat# A0008, RRID:AB_2650473
	Mouse anti-SMA	1:200	Abcam, Cat# ab7817, RRID:AB_262054
Secondary antibodies	Goat Anti-Mouse555	1:1000	Thermo Fisher, Cat#A-21422, RRID:AB_2535844
	Goat Anti-Rabbit555	1:1000	Thermo Fisher, Cat# A11034, RRID:AB_2576217
	Goat Anti-Mouse488	1:1000	Thermo Fisher, Cat# A11029, RRID:AB_138404
Flow cytometry	Human anti-TRA-1-60-PE	1:11	Miltenyi Biotec, Cat#130-100-347, RRID:AB_2654227
	Human anti-SSEA-4-APC	1:11	Miltenyi Biotec, Cat# 130-098-347, RRID:AB_2653520

Primers		
	Target	Forward/Reverse primer sequence (5′–3′)
Sendai virus detection	iPSC-SeV-F	ggatcactaggtgatatcgagc
	iPSC-SeV-R	accagacaagagtttaagagatatgtatc
	iPSC-KOS-F	atgcaccgctacgacgtgagcgc
	iPSC-KOS-R	accttgacaatcctgatgtgg
	iPSC-Klf4-F	ttcctgcatgccagaggagccc
	iPSC-Klf4-R	aatgtatcgaaggtgctcaa
	iPSC-c-Myc-F	taactgactagcaggcttgtcg
	iPSC-c-Myc-R	tccacatacagtcctggatgatgatg

markers; Sigma) for 30 min at room temperature. Primary and secondary antibodies (Table 2) together with DAPI (300 nM; Sigma) were incubated 12 h at 4 °C and 1 h at room temperature, respectively. Cells were visualized under an inverted fluorescence microscope (Olympus).

STR analysis

STR analysis was conducted by CLS Cell Lines Service GmbH.

Array-CGH

Sample and reference DNA were labeled with Cy3 and Cy5 and hybridized on a 400 k whole genome array (SurePrint G3 Human Genome CGH Microarray, Agilent Technologies). Scan data were processed using Agilent Feature Extraction software and open source software (GenomeCAT) (Tebel et al., 2017). Copy-number aberrations were detected using the circular binary segmentation algorithm (Venkatraman and Olshen, 2007).

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Fig. 1. Characterization of *SLC2A3*-D2-iPSCs. (A–B) Generation of iPSCs from patient's skin fibroblasts. (C) Karyogram of a normal female karyotype (46, XX). (D) Flow cytometric analysis of pluripotency markers TRA-1-60 and SSEA-4. (E) Duplicated region comprising the *SLC2A3* gene locus identified by Array-CGH (indicated by a red bar). (F) Immunostaining of pluripotency markers TRA-1-60, SSEA-4 and OCT-3/4, nuclei stained with DAPI. (G) Elimination of non-integrating viral genome. (H) Mycoplasma detection. (I) iPSCs form embryoid bodies and (J) differentiate into cells of all three germ layers (Mesoderm: SMA; Endoderm: AFP; Ectoderm: ßTubIII; nuclei stained with DAPI).

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