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Lab Resource: Multiple Cell Lines

Generation of induced pluripotent stem cell lines AKOSi002-A and AKOSi003-A from symptomatic female adults with Wilson disease

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A B S T R A C T

Wilson disease (WD) is an inherited, autosomal recessive disorder of copper metabolism caused by mutations in the *ATP7B* gene. Pathogenic single nucleotide variants (SNVs) lead to functional impairment of the copper transporting ATPase ATP7B, resulting in copper accumulation and toxicity in the liver and brain. We describe the generation of two induced pluripotent stem cell (iPSC) lines derived from fibroblasts of two female WD patients. Patient 1 is compound heterozygous for p.E1064A and p.H1069Q. Patient 2 is homozygous for p.M769V. These iPSCs represent a WD model for pathophysiological studies and pharmacological screening.

Resource table

Unique stem cell lines identifier	1: AKOSi002-A2: AKOSi003-A
Alternative names of stem cell lines	1: iPS GM05257-11 2: iPS F0101990-1
Institution	Translational Neurodegeneration Section "Albrecht-Kossel", Department of Neurology, University Medical Center Rostock, University of Rostock, 18147 Rostock, Germany
Contact information of distributor	Dr. Jan Lukas; jan.lukas@med.uni-rostock.de
Type of cell lines	iPSC
Origin	Human
Cell source	Fibroblasts
Clonality	Clonal
Method of reprogramming	Retrovirus
Multiline rationale	Same disease non-isogenic cell lines
Gene modification	Yes
Type of modification	Hereditary
Associated disease	Wilson disease
Gene/locus	1: *ATP7B* / 13q14.3c.3191A>C/c.3207C>A 2: *ATP7B* / 13q14.3c.2305A>G/c.2305A>G
Method of modification	N/A
Name of transgene or resistance	OCT4, SOX2, KLF4, C-MYC
Inducible/constitutive system	N/A
Date archived/stock date	April 2018
Cell line repository/bank	N/A

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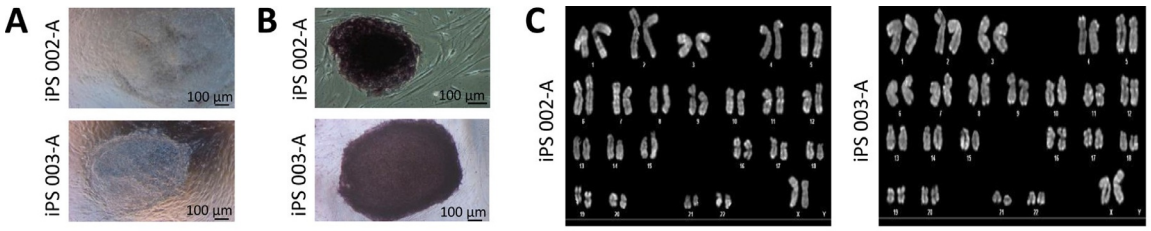
Ethical approval

Fibroblasts GM05257 were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research, USA. Fibroblasts F0101990 were obtained from the "Cell Line and DNA Biobank from Patients Affected by Genetic Diseases", member of the Telethon Network of Genetic Biobanks, Italy



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Cell line	Gene	Amino Acid Change	Allele Coverage	Allele Ratio	% Frequency
iPS 002-A	ATP7B	p.His1069Gln	G=952, T=1047	G=0.4762, T=0.5238	52.38
iPS 002-A	ATP7B	p.Glu1064Ala	T=1059, G=936	T=0.5308, G=0.4692	46.92
iPS 003-A	ATP7B	p.Met769Val	T=1, C=1989	T=5.0E-4, C=0.9995	99.95

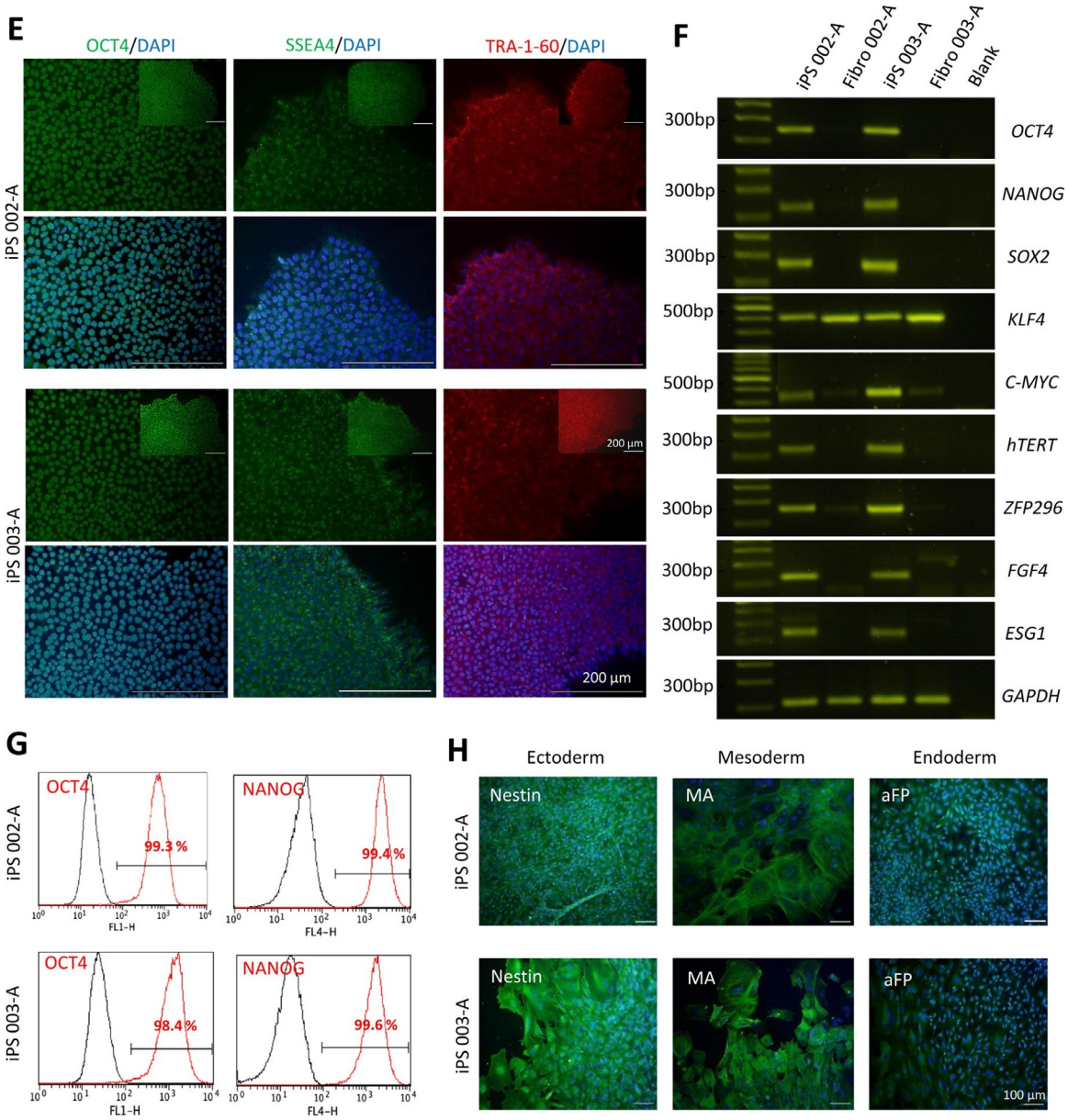

Fig. 1. Characterization of iPSCs AKOSi002-A and AKOSi003-A from two female patients with Wilson disease.

Table. 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
AKOSi002-A (iPS GM05257-11)	iPS 002-A	Female	34	Caucasian	NM_000053.4:c.3191A > C/NM:000053.4:c.3207C > A	Wilson disease
AKOSi003-A (iPS F0101990-1)	iPS 003-A	Female	unknown	unknown	NM_000053.4:c.2305A > G/hom	Wilson disease

Table. 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Visual record of the lines: normal	Fig. 1 panel A
Phenotype	Qualitative analysis: Alkaline phosphatase staining	Positive	Fig. 1 panel B
	Qualitative analysis: Immunocytochemistry	Expression of pluripotency markers: OCT4, NANOG, SSEA4, TRA-1-60, TRA-1-81	Fig. 1 panel E (representative)
	Qualitative analysis: RT-PCR	Expression of pluripotency genes: *OCT4, NANOG, SOX2, KLF4, C-MYC, hTERT, ZFP296, FGF4, ESG1*	Fig. 1 panel F
	Quantitative analysis: Flow cytometry	Percentage of positive cells iPS 002-A: OCT4: 99.3%, NANOG: 99.4%, SSEA4: 99.5%, TRA-1-60: 92.8%, TRA-1-81: 81.8% iPS 003A: OCT4: 98.4%, NANOG: 99.6%, SSEA4: 99.4%, TRA-1-60: 91.4%, TRA-1-81: 91.4%	Fig. 1 panel G (representative)
Genotype	Karyotype (G-banding) and resolution	46, XX Resolution 150–300	Fig. 1 panel C
Identity	Microsatellite PCR (mPCR) OR	Not performed	N/A
	STR analysis	18 STR loci tested, 100% matched	Available with authors
Mutation analysis (IF APPLICABLE)	Sequencing	iPS 002-A: compound heterozygous p.E1064A/p.H1069Q iPS 003-A: homozygous p.M769V	Fig. 1 panel D
	Southern Blot OR WGS	Not performed	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR: Negative	Supplementary Fig. S1 panel B
Differentiation potential	Embryoid body formation	Expression of genes in embryoid bodies: Muscle actin (MA), nestin and α-fetoprotein (aFP)	Fig. 1 panel H
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	N/A
	HLA tissue typing	Not performed	N/A

1. Resource utility

Wilson disease is a rare monogenetic disorder due to mutations in the *ATP7B* gene. To reflect the genotypic and phenotypic variability of the disease, the establishment of a patient-specific cell model can aid to improve our understanding of the underlying pathophysiological mechanisms and can be used in future drug evaluation.

2. Resource details

Wilson disease (WD) is a rare, genetic copper storage disorder caused by pathogenic homozygous or compound heterozygous mutations in the *ATP7B* gene. Currently, almost 800 *ATP7B* variants were reported to be causative for WD (Medici and LaSalle, 2019).

In this study, skin fibroblasts from two female WD patients carrying the most common European variants, p.H1069Q and p.M769V (Coffey et al., 2013), were reprogrammed to obtain patient-specific iPSC lines. GM05257 (Fibro 002-A, Coriell Institute for Medical Research) was compound heterozygous for p.E1064A/p.H1069Q, while F0101990 (Fibro 003-A, Cell Line and DNA Biobank from Patients Affected by Genetic Diseases Italy (Filocamo et al., 2014) was homozygous for p.M769V. Genetic analysis confirmed the presence of the pathogenic SNVs prior to reprogramming using Sanger sequencing (data not shown). Retroviral transduction of the fibroblasts resulted in typical iPSC colony formation with embryonic stem cell-like morphology (Fig. 1A). Absence of retroviral activity in the iPSCs was confirmed by loss of GFP signal (Supplementary Fig. S1A). Colonies were picked and passaged as clonal lines on irradiated mouse embryonic fibroblasts (MEF) before they were transferred onto Matrigel-coated plates. Clones AKOSi002-A (iPS 002-A) and AKOSi003-A (iPS 003-A) were selected for further characterization (Table 1 and 2). The alkaline phosphatase (AP) activity test showed an intense purple staining as a result of high AP activity in the iPSC lines. No AP activity could be observed in the surrounding MEF (Fig. 1B). Chromosome analysis revealed a normal female karyotype (46, XX) for both iPSC lines (Fig. 1C). Mutation analysis using targeted NGS sequencing confirmed the genetic mutation of parental fibroblasts in both iPS 002-A and iPS 003-A (Fig. 1D). Short tandem repeat analysis of 18 genomic loci showed exact matches of all tested alleles between the parental fibroblasts and the corresponding iPSC line (excerpt submitted in archive with journal). Immunocytochemistry and flow cytometry analysis demonstrated high expression of intracellular and surface markers of pluripotency, e.g. OCT4, NANOG, SSEA4 and TRA-1-60 (Fig. 1E and 1G). Furthermore, RT-PCR for pluripotency-related genes was performed. Compared to the parental fibroblasts, the iPSC lines showed distinct gene expression of *OCT4, NANOG, SOX2, C-MYC, hTERT, ZFP296, FGF4* and *ESG1* (Fig. 1F). Primer pairs used for *OCT4, SOX2, KLF4* and *C-MYC* amplified endogenous but not transgenic transcripts. *GAPDH*was used as technical control. Spontaneous differentiation potential into cells of all three germ layers was demonstrated by in vitro formation of embryoid bodies. Immunocytochemistry confirmed expression of nestin (ectoderm), muscle actin (MA, mesoderm) and alpha-fetoprotein (aFP, endoderm) in both iPS 002-A and iPS 003-A (Fig. 1H). Additionally, the iPSC lines were tested negative for mycoplasma contamination (Supplementary Fig. S1B).

3. Materials and methods

3.1. Reprogramming of fibroblasts

Patient-derived fibroblasts were reprogrammed using retroviral vectors for OCT4, SOX2, KLF4 and C-MYC following the protocol previously published by Peter et al. (2017).

3.2. Cell culture

Fibroblasts and MEF feeder cells were cultured in high glucose DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS, GE Healthcare) and 1% penicillin-streptomycin (10,000 U/ml, Gibco). IPSCs cultured on feeder cells were kept in iPSC medium (DMEM/F12, 20% Knockout serum replacement, 0.1 mM MEM non-essential amino acids, 1% GlutaMax, 0.1 mM 2-mercaptoethanol, 1% penicillin–-streptomycin (all Gibco) supplemented with 12.5 ng/ml FGF-2 (Amsbio)). Passaging of iPSCs on feeder cells was done mechanically with glass hooks. IPSC colonies cultured on Matrigel (Corning) were maintained in mTeSR1 (STEMCELL Technologies) and 0.25% penicillin-streptomycin (Gibco). Feeder cell-free iPSCs on Matrigel were passaged using Gentle Cell Dissociation Reagent (STEMCELL Technologies). Cells were incubated at 37 °C in a humidified 5% CO_2 incubator.

3.3. Alkaline phosphatase activity test

IPSC colonies were fixed in cold methanol and incubated with staining solution (75% distilled water, 10% 1 M sodium chloride, 10% 1 M Tris (pH 9.8), 5% 1 M magnesium chloride, 1:50 NBT/BCIP stock solution (Roche) for 15 min.

3.4. Karyotyping

After 24 h incubation with Colcemid (0.02 ng/ml) at 37 °C, metaphases of the iPSC lines in passages 14 and 15 were harvested. After centrifugation, the cells were treated with 0.075 M potassium chloride, sedimented and fixed in methanol–acetic acid (3:1). Ten metaphase spreads for every iPSC line were counted. Chromosome analysis was performed using RC-banding technique according to standard procedures in routine diagnostics implemented in the Department of Hematology, University Medical Center Rostock.

3.5. Immunocytochemistry

IPSC colonies were seeded onto Matrigel-coated glass cover slips. After 3–5 days, the cells were fixed in 4% paraformaldehyde for 15 min. Blocking (10% normal goat serum (NGS), 0.1% Triton-X 100 in PBS) was performed for 45 min at room temperature before primary antibody incubation overnight at 4 °C and secondary antibody incubation for 1 h at room temperature. Finally, DAPI was added for 5 min at room temperature. Between each antibody incubation, extensive washing was performed. For a list of used antibodies refer to Table 3. A digital compact microscope (Keyence) was used for imaging.

3.6. RT-PCR

Total RNA was extracted using Quick-RNA Miniprep kit (Zymo Research). The quality of isolated total RNA was determined by TAE agarose gel electrophoresis and nucleic acid concentration was measured in a multifunction reader equipped with NanoQuant plate (Tecan). One-step Reverse Transcriptase PCR (QIAGEN) was performed in an Eppendorf 5331 MasterCycler Gradient Thermal Cycler. 100 ng of total RNA were used for each reaction. Cycle number and annealing temperatures were optimized for each primer pair. For primer sequences refer to Table 3. PCR products were run on TBE agarose gels.

3.7. Flow cytometry

Cells were harvested and incubated with fluorophore-conjugated

Table. 3
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Marker (IF)	Rabbit anti-OCT4	1:100	Stemgent Cat# 09-0023, RRID: AB_2167689
Pluripotency Marker (IF)	Rabbit anti-NANOG	1:100	Stemgent Cat# 09-0020, RRID: AB_2298294
Pluripotency Marker (IF)	Mouse anti-SSEA4	1:100	Stemgent Cat# 09-0006, RRID: AB_1512169
Pluripotency Marker (IF)	Mouse anti-TRA-1-60	1:100	Stemgent Cat# 09-0010, RRID: AB_1512170
Pluripotency Marker (IF)	Mouse anti-TRA-1-81	1:100	Stemgent Cat# 09-0011, RRID: AB_1512171
Pluripotency Marker (FC)	Alexa Fluor 488 anti-OCT4, mouse IgG2b	1:20	BioLegend Cat# 653705, RRID: AB_2562250
Pluripotency Marker (FC)	Alexa Fluor 647 anti-NANOG, mouse IgG1	1:50	BioLegend Cat# 674210, RRID: AB_2650619
Pluripotency Marker (FC)	Alexa Fluor 647 anti- SSEA-4, mouse IgG3	1:500	BioLegend Cat# 330407, RRID: AB_1089201
Pluripotency Marker (FC)	PE anti-human TRA-1-60-R, mouse IgM	1:20	BioLegend Cat# 330609, RRID: AB_1279447
Pluripotency Marker (FC)	Alexa Fluor 488 anti-TRA-1-81, mouse IgM	1:20	BioLegend Cat# 330709, RRID: AB_2561741
Differentiation Marker (IF)	Mouse anti-Muscle actin	1:50	Agilent Dako Cat# M0635, RRID: AB_2242301
Differentiation Marker (IF)	Mouse anti-Nestin	1:100	R and D Systems Cat# MAB1259, RRID: AB_2251304
Differentiation Marker (IF)	Mouse anti-Alpha fetoprotein	1:20	R and D Systems Cat# MAB1368, RRID: AB_357658
Secondary antibody	Alexa Fluor 488, Goat anti-mouse IgG	1:500	Thermo Fisher Scientific Cat# A-11029, RRID: AB_2534088
Secondary antibody	Alexa Fluor 568, Goat anti-mouse IgM	1:500	Thermo Fisher Scientific Cat# A-21043, RRID: AB_2535712
Secondary antibody	Alexa Fluor 488, Goat anti-rabbit IgG	1:500	Thermo Fisher Scientific Cat# A-11034, RRID: AB_2576217

Primers		
	Target	Forward/Reverse primer (5′−3′)
Pluripotency Marker (RT-PCR)	*C-MYC*	GCGTCCTGGGAAGGGAGATCCGGAGC/ TTGAGGGGCATCGTCGCGGGAGGCTG
Pluripotency Marker (RT-PCR)	*NANOG*	TGTGTTCTCTTCCACCCAGC/ACCAGGTCTTCACCTGTTTGT
Pluripotency Marker (RT-PCR)	*OCT4*	GACAGGGGGAGGGGAGGAGCTAGG/ CTTCCCTCCAACCAGTTGCCCCAAAC
Pluripotency Marker (RT-PCR)	*SOX2*	AGGGAGAGAAGTTTGAGCCC/GCGAGGAAAATCAGGCGAAG
Pluripotency Marker (RT-PCR)	*KLF4*	ACGATCGTGGCCCCGGAAAAGGACC/ TGATTGTAGTGCTTTCTGGCTGGGCTCC
Pluripotency Marker (RT-PCR)	*ZFP296*	CTGGACCGACAAACACCCAG/CTTCAGCTCCTCTCGTTCTGAG
Pluripotency Marker (RT-PCR)	*ESG1*	ATATCCCGCCGTGGGTGAAAGTTC/ ACTCAGCCATGGACTGGAGCATCC
Pluripotency Marker (RT-PCR)	*FGF4*	CAAGCTCTATGGCTCGCCCT/TCTTCCCATTCTTGCTCAGGG
Pluripotency Marker (RT-PCR)	*hTERT*	GAGCTGACGTGGAAGATGAGC/CATCAGCCAGTGCAGGAACTT
House-Keeping Gene (RT-PCR)	*GAPDH*	CATGTTCCAATATGATTCCACCC/GGGATCTCGCTCCTGGAAGAT

antibodies for 1 h at room temperature. 5×10^4 cells were analyzed using FACSCalibur (BD). Fixation and permeabilization for intracellular staining of iPSCs were done using True-Nuclear™ Transcription Factor Buffer Set (BioLegend). Data analysis was performed with software FCSalyzer version 0.9.18-alpha (https://sourceforge.net/projects/fcsalyzer/).

3.8. Embryoid body (EB) formation

IPSC colonies were mechanically detached from the feeder cell layer, transferred to a low attachment plate and incubated at 37 °C and 5% CO_2. IPSC medium was changed to EB medium containing 78% Knockout DMEM, 0.1 mM MEM non-essential amino acids, 1% GlutaMax, 0.1 mM 2-mercaptoethanol, 0.25% penicillin-streptomycin (all Gibco) and 20% FBS (GE Healthcare). Floating colonies were seeded onto gelatin-coated cover slips after 5 days. EBs were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton-X 100 and separately blocked with 4% NGS after 9 days of differentiation.

3.9. Short tandem repeat (STR) analysis

18 STR loci were analyzed at ATCC, VA, USA. Cell suspensions of fibroblasts and iPSCs (1×10^6 cells/ml) were collected on Whatman® FTA® cards according to the manufacturer's protocol and shipped to ATCC.

3.10. Targeted sequencing

Genomic DNA was extracted (Quick-DNA™ Miniprep Kit, Zymo Research) and quantified using a multifunction reader (Tecan). Targeted sequencing library construction was performed using a custom designed Ion AmpliSeq™ ATP7B Panel (Thermo Fisher Scientific). 10 ng of genomic DNA were used to amplify the complete coding sequence. Sequencing was carried out on an Ion Torrent™ Personal Genome Machine™ System, using an Ion Torrent 318 V2 chip. Sequence check was performed against the hg19 assembly of the human genome using Torrent Suite™ software and the variant caller plugin version 5.10.0.18 (Thermo Fisher Scientific).

3.11. Mycoplasma detection

Mycoplasma detection was performed using PCR Mycoplasma Test Kit I/C (PromoCell) following manufacturer's instructions.

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Declaration of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2020.101708.

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