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Generation of two induced pluripotent stem cell lines from peripheral blood mononuclear cells of a patient with Wilson's disease

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A B S T R A C T

Wilson's disease is an inherited disorder associated with copper accumulation in the liver, brain and other vital organs. Wilson's disease is caused by mutations in the *ATP7B* gene. Over 300 mutations of *ATP7B* have been described. Despite the disease is autosomal recessive, the patient whose PBMCs were reprogrammed in the study harbours heterozygous mutation c.3207C > A (p.H1069Q). Detailed analysis of the *ATP7B* complete gene sequencing data has not revealed other known disease associated mutation. The generated iPSC lines maintained the original genotype, expressed pluripotency markers, had normal karyotype and demonstrated the ability to differentiate into derivatives of the three germ layers.

Resource Table:

Unique stem cell lines identifier	ICGi020-A ICGi020-B
Alternative names of stem cell lines	ATP7bIL23f ATP7bIL24f
Institution	Federal Research Center Institute of Cytology and Genetics, the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
Contact information of distributor	Anastasia A. Malakhova amal@bionet.nsc.ru
Type of cell lines	iPSC
Origin	Human
Cell Source	PBMCs
Clonality	Clonal
Method of reprogramming	Episomal plasmid vectors
Multiline rationale	Isogenic clones
Gene modification	YES
Type of modification	Hereditary mutation
Associated disease	Wilson's disease
Gene/locus	13q14.3 ATP7B:c.3207C > A
Method of modification	N/A N/A
Name of transgene or resistance	
Inducible/constitutive system	N/A
Date archived/stock date	April 2020
Cell line repository/bank	https://hpscreg.eu/user/cellline/edit/ICGi020-A https://hpscreg.eu/user/cellline/edit/ICGi020-B
Ethical approval	The study has been approved by the Research Ethics Committee of the Research Institute of Medical Genetics of the Tomsk National Research Medical Center (Tomsk, Russia), protocol number 125/1 (22.10.2018)

1. Resource utility

Wilson's disease is characterized by significant clinical polymorphism, the nature of which remains unclear (Merle et al., 2007). The patient-specific iPSC lines are useful for modelling the disease *in vitro* and searching for modifier genes that cause the phenotypic heterogeneity. Studying pathogenetic copper metabolism pathways in hepatocyte-like cells derived from the iPSCs is helpful for discovering targeted therapy of the chronic progressive disease.

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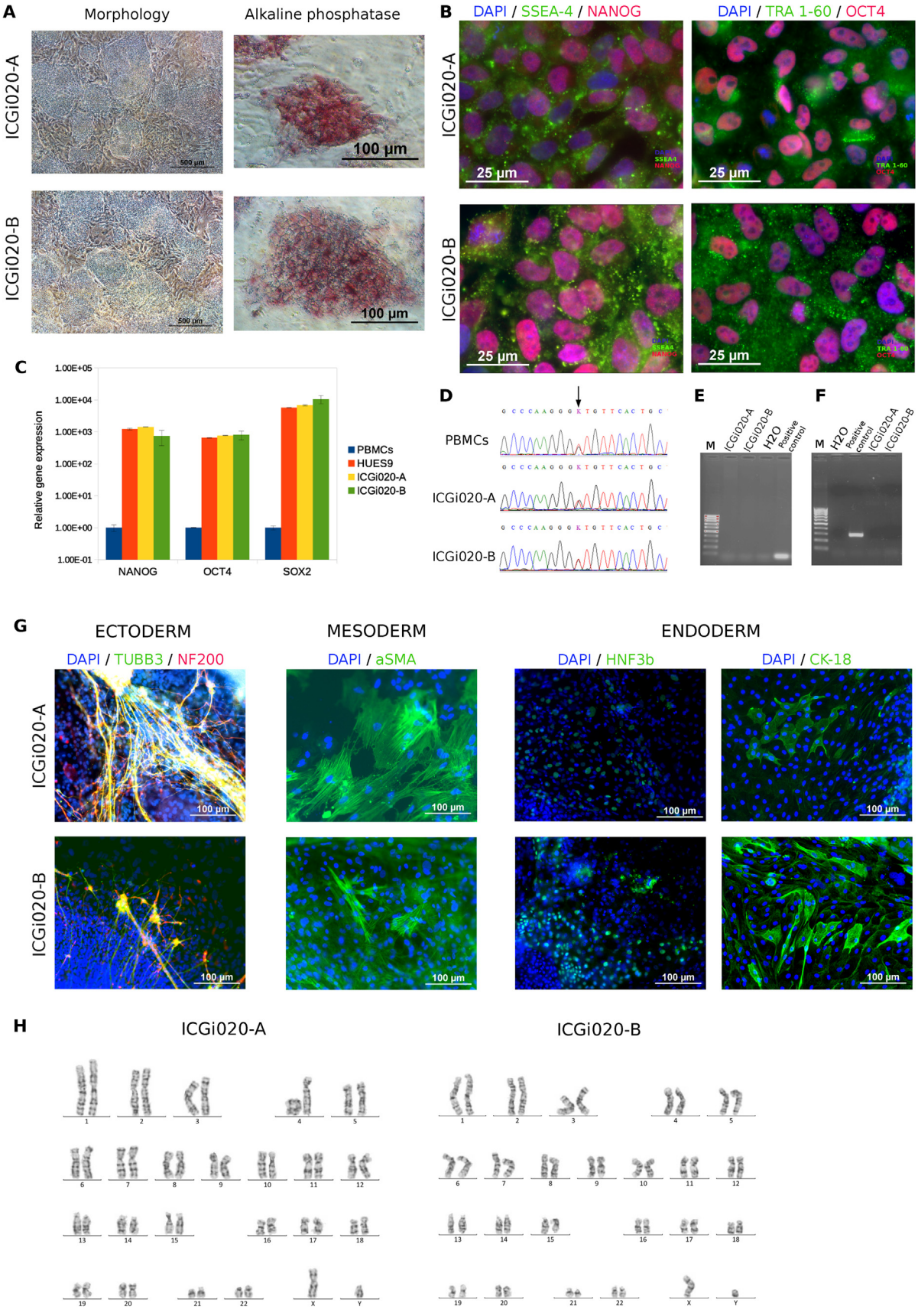

Fig. 1. Characterization of the ICGi020-A and ICGi020-B iPSC lines.

2. Resource details

The peripheral blood mononuclear cells (PBMCs) were donated by a 16-year-old man with Wilson's disease symptoms. Targeted NGS of the *ATP7B* complete gene sequence revealed heterozygous substitution c.3207C > A. PBMCs were reprogrammed to pluripotent state via transfection by integration free episomes encoding pluripotency factors OCT4, SOX2, KLF4, L-MYC, LIN28 and p53 shRNA (Okita et al., 2013). The established ICGi020-A and ICGi020-B iPSC lines demonstrated typical pluripotent cell morphology in phase contrast microscopy and expressed alkaline phosphatase (Fig. 1A). The cells were positive for pluripotency markers such as transcription factors OCT4 and NANOG, and surface markers SSEA-4 and TRA-1-60 (Fig. 1B). A quantitative real-time PCR (qPCR) showed high levels of *OCT4*, *SOX2* and *NANOG* expression in the ICGi020-A and ICGi020-B cell lines as well as in the human embryonic stem cell line HUES9 (HVRDe009-A) (Cowan et al., 2004), as compared to the primary PBMCs (Fig. 1C). The presence of the disease associated mutation in the iPSC lines was confirmed by Sanger sequencing (Fig. 1D). Elimination of the episomal reprogramming vectors was confirmed by PCR (Fig. 1E). ICGi0120-A and ICGi020-B cells were also shown to be free of mycoplasma contamination (Fig. 1F). The ability to generate derivatives of the three-germ layers was shown by spontaneous *in vitro* differentiation through formation of embryoid bodies. Immunofluorescent staining revealed ectodermal (Neurofilament 200 (NF200) and Tubulin beta 3 (TUBB3)), endodermal (Cytokeratin 18 (CK-18) and Hepatocyte nuclear factor HNF3b), and mesodermal (Smooth muscle actin alpha (αSMA)) markers among the differentiated cells (Fig. 1G). Short tandem repeats (STR) analysis of the ICGi020-A and ICGi020-B lines demonstrated an identical DNA profile at 20 polymorphic loci with the primary PBMCs (data available with the authors). Karyotype analysis (G-banding) revealed normal male chromosomal set (46,XY) after 15 passages in culture (Fig. 1H). Full characterization is summarized in Table 1

3. Materials and methods

3.1. PBMCs reprogramming

PBMCs were reprogrammed using a set of episomal vectors encoding *OCT4*, *KLF4*, *L-MYC*, *SOX2*, *LIN28* and *mp53DD* (Addgene IDs #41855–58, #41813–14). 5 × 10^5 of cells were transfected with 500 ng of each vector using Neon Transfection System (Thermo Fisher Scientific) and handled as described in Epi5™ Episomal iPSC Reprogramming Kit user guide (Thermo Fisher Scientific) for obtaining feeder-dependent iPSCs. The primary iPSC colonies were picked manually and plated onto mitotically inactivated MEFs in KnockOut DMEM with 15% KoSR, 0.1 mM NEAA, 0.1 mM 2-mercapthoethanol, 100 U/ml penicillin–streptomycin, 2 mM GlutaMAX and 10 ng/ml bFGF. For passaging iPSCs were dissociated with TrypLE (Life Technologies) and split at 1:10 every 4–5 days. Cells were maintained at 37 °C in 5% CO_2.

3.2. DNA isolation

DNA isolation was performed using Quick-DNA Miniprep Kit (Zymo Research).

3.3. Mutation analysis

To analyze the mutations in the *ATP7B* gene, a primer panel was developed for complete gene sequencing using targeted NGS (Table 3). The enrichment of the genome regions of interest was carried out using long-range PCR with BioMaster LR HS-PCR-Color (2x) kit (Biolabmix). Amplification was carried out on a SureCycler 8800 using the following program: 94 °C for 4 min; 10 cycles: 94 °C 20 s, 57 °C 30 s, 68 °C 13 min; 20 cycles: 94 °C 20 s, 57 °C 30 s, 68 °C 13 min (+10 s per cycle). DNA libraries were prepared using the Nextera Flex kit (Illumina). NGS was performed on a MiSeq sequencer using the MiSeq Reagent Kit V2 sequencing kit (Illumina).

The identified mutation was confirmed by Sanger sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) on an ABI Prism 3730 capillary sequencer (Applied Biosystems).

3.4. Immunofluorescence staining

Cells growing on 24-well plate were fixed in 4% paraformaldehyde for 10 min at room temperature, permeabilized in 0.5% Triton-X100 for 30 min, then incubated with Blocking Buffer (1% BSA in PBS) for 30 min. Fixed cells were incubated with primary antibodies overnight at 4 °C, washed twice with PBS and incubated with secondary antibody for 1.5–2 h at RT (Table 2). All antibodies were diluted in PBS with 1% BSA. Nuclei were counterstained with DAPI (Sigma). Micrographs were taken using Nikon Eclipse Ti-E microscope and NIS Elements software.

3.5. RT-qPCR

RNA was isolated using Trizol (Thermo Fisher Scientific). Reverse transcription was performed using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). qRT-PCR reactions were run on a LightCycler 480 Real-Time PCR System (Roche) with BioMaster HS-qPCR SYBR Blue 2× (Biolabmix). CT-values were normalized to *b-ACTIN* using ΔΔCT-method.

3.6. In vitro spontaneous differentiation

iPSCs were detached with 0.15% Type IV Collagenase (Thermo Fisher Scientific), seeded onto 100 mm dishes coated with 1% agarose in DMEM/F-12 supplemented with 10% FBS, 0.1 mM NEAA, 2 mM GlutaMax, 100 U/ml penicillin–streptomycin and cultivated for 14 days. Then embryoid bodies were plated onto gelatin-treated 24-well plate for another 14 days.

3.7. Karyotype analysis

Cells were evaluated at passage 15. Karyotyping was performed in the Research Institute of Medical Genetics Clinic (Tomsk, Russia) using 450–500 G-banding chromosome analysis. 20 metaphase plates were analyzed using Ikaros software (MetaSystems).

3.8. Mycoplasma and episomes detection

Detection of mycoplasma and *EBNA* sequence was performed using PCR (95 °C 5 min; 35 cycles: 95 °C 15 s, 60 °C 15 s, 72 °C 20 s) thermocycler C1000 (Bio-Rad) as described earlier (Choppa et al., 1998;

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
ATP7bIL23f (ICGi020-A)	ICGi020-A	Male	16	Caucasian	*C/A*	Wilson's disease
ATP7bIL24f (ICGi020-B)	ICGi020-B	Male	16	Caucasian	*C/A*	Wilson's disease

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Phase contrast microscopy	Normal	Fig. 1 panel A
Phenotype	Qualitative analysis *Immunofluorescence staining*	Positive staining for pluripotency markers: *NANOG, OCT4, SOX2, TRA-1–60*	Fig. 1 panel B
	Quantitative analysis by *RT-qPCR*	Expression of pluripotency markers: *NANOG, OCT4, SOX2*	Fig. 1 panel C
Genotype	Karyotype (G-banding) and resolution	46,XY Resolution 450–500	Fig. 1 panel H
Identity	STR analysis	20 of 20 sites were matched	Data available with the authors
Mutation analysis	Sanger sequencing	Heterozygous mutation c.3207C > A in the *ATP7B* gene	Fig. 1 panel D
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR - Negative	Fig. 1 panel F
Differentiation potential	Embryoid body formation	Positive staining for germ layer markers: αSMA (mesoderm); NF200 and TUBB3 (ectoderm); CK-18 and HNF3b (endoderm)	Fig. 1 panel G
Donor screening (OPTIONAL)	N/A	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 3
Reagents details.

Antibodies used for immunocytochemistry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Rabbit IgG anti-OCT4	1:200	Abcam Cat# ab18976, RRID:AB_444714
	Rabbit IgG anti-NANOG	1:200	Abcam Cat # ab62734, RRID: AB_956161
	Mouse IgG3 anti-SSEA4	1:200	Abcam Cat # ab16287, RRID:AB_778073
	Mouse IgM anti-TRA-1–60	1:200	Abcam Cat # ab16288, RRID:AB_778563
Differentiation Markers	Mouse IgG2a anti-αSMA	1:100	DAKO Cat # M0851, RRID: AB_2223500
	Rabbit IgG anti-NF200	1:1000	Sigma Cat # N4142, RRID: AB_477272
	Mouse IgG2a anti-TUJ1	1:500	BioLegend Cat # 801201 RRID:AB_2313773
	Mouse IgG1 anti-HNF3b	1:50	Santa Cruz Biotechnology Cat #sc-374376 RRID:AB_10989742
	Mouse IgG1 anti-CK18	1:200	Millipore Cat # MAB3234, RRID:AB_94763
Secondary antibodies	Goat anti-Rabbit IgG (H + L) Alexa Fluor 568	1:400	Thermo Fisher Scientific Cat # A11011 RRID:AB_143157
	Goat anti-Mouse IgG (H + L) Secondary Antibody, Alexa Fluor 488	1:400	Thermo Fisher Scientific Cat # A11029 RRID:AB_2534088
Primers			
	Target		Forward/Reverse primer (5′-3′)
Episomal plasmid vectors detection	*EBNA-1*		GCCAATGCAACTTGGACGTT/ATCAGGGCCAAGACATAGAGATG
House-keeping gene (RT-qPCR)	*bACTIN*		GCACAGAGCCTCGCCTT/ GTTGTCGACGACGAGCG
Pluripotency marker (RT-qPCR)	*NANOG*		CAGCCCCGATTCTTCCACCAGTCCC/ CGGAAGATTCCCAGTCGGGTTCACC
Pluripotency Marker (qPCR)	*OCT4*		CTTCTGCTTCAGGAGCTTGG/ GAAGGAGAAGCTGGAGCAAA
Pluripotency Marker (qPCR)	*SOX2*		GCTTAGCCTCGTCGATGAAC/ AACCCCAAGATGCACAACTC
Mycoplasma detection	16S ribosomal RNA gene		GGGAGCAAACAGGATTAGATACCCT/ TGCACCATCTGTCACTCTGTTAACCTC
ATP7B mutation detection	*ATP7B Coordinates (hg38)*	chr13:51973844-51981403	GAAAGTGGAGCGAGTGTGGA/ AGCAGGGCTCACCTATACCA
		chr13:51964406-51969914	AATTCTCACGGATTTTCCAAAGCAG/ TCTCTTTTCTTACCCCAGTGATGTG
		chr13:51967821-51974541	TCAAACATAGCATGTTCTAGGCATC/ TTACAAAGCACTAACCCAAAGAGAC
		chr13:51939724-51948005	TTTTTCGGGAAAGCAGTGCG/ CATACGAGAGGGCACGACTC
		chr13:51932933-51939493	GTCATACGTGCTCCTTGCAG/ CAATTACTGACGGACAGCGG
		chr13:52006868-52012469	TGCTCACCTCAACAACTTGC/ CCACCTCCCACTGAAGGAAT
		chr13:51931715-51937055	CTAGTGGAGATGCTTGGCTG/ CAATGGGACATGCACACAAC
		chr13:51956648-51964995	AGAGTCCAAACTCACGAGGA/ CAGTAGTCCAAAGCGAGACC
		chr13:51953343-51958532	TGGTACTTCTACGTTCAGGC/ ACTAAAGGTCAGACCCTCCT
		chr13:51946315-51957768	TGGTGGATAGCAAGTAACGC/ TTGATGAGGATGCCGTTCTG

Okita et al., 2013). Primers are listed in Table 2.

3.9. STR profiling

Cell identity was assessed on genomic DNA from hiPSC line and genomic DNA from blood with the Promega PowerPlex Fusion System. 20 STR markers were analyzed.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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