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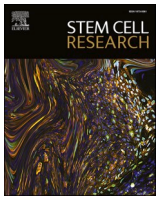

Lab Resource: Multiple Cell Lines

Generation of induced pluripotent stem cells from two ADHD patients and two healthy controls

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A B S T R A C T

Attention-deficit hyperactivity disorder is a neurodevelopmental disorder which prevalence has been increasing in the past decades, affecting more than 5% of children, adolescents worldwide. Regarding etiology, polygenic, environmental factors contribute to the occurrence of ADHD even though molecular mechanisms are not known. Understanding the pathophysiology in patient-specific cells is crucial for the discovery of potential predictive markers, the establishment of new therapeutic targets. In this study, we generated further lines from ADHD patients, healthy controls using Sendai virus transduction, which may help on the study of ADHD at the molecular, cellular levels.

Resource Table:

Unique stem cell lines identifier	TMPi010-A TMPi010-B TMPi011-A TMPi011-B TMPi008-B TMPi012-A TMPi012-B
Alternative name(s) of stem cell lines	Healthy controls (“KO” = “Control”):KO-013 c20 (TMPi010-A)KO-013 c39 (TMPi010-B)KO-015 c1 (TMPi011-A)KO-015 c9 (TMPi011-B) ADHD lines (“MR” = “Methylphenidate Responder”) :MR-013 c13 (TMPi008-B)MR-023 c17

(*continued on next page*)

Abbreviations: PBMC, Peripheral blood mononuclear cell; ADHD, Attention-deficit hyperactivity disorder; SeV, Sendai virus; CNV, Copy Number Variation; EBs, embryoid bodies; MR, Methylphenidate responder; KO, Control.

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(*continued*)

	(TMPi012-A)MR-023 c22 (TMPi012-B)
Institution	Psychiatric University Hospital Zurich, Department of Child and Adolescent Psychiatry and Psychotherapy, University of Zurich.
Contact information of distributor	Prof. Dr. Edna Grünblatt (edna.gruenblatt@kjpd.uzh.ch)
Type of cell lines	iPSC
Origin	Human
Additional origin info required for human ESC or iPSC	Age: MR013: 16 years old MR023: 12 years old K013: 9 years old K015: 13 years old Sex: MR013: male MR023: male K013: female K015: male Ethnicity: all individuals have Caucasian origin
Cell Source	Peripheral Blood Mononuclear Cells
Clonality	Clonal
Method of reprogramming	Sendai virus transduction
Genetic Modification	NO
Type of Genetic Modification	N/A
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	N/A
Associated disease	Attention-Deficit Hyperactivity Disorder (ADHD) and healthy controls
Gene/locus	N/A
Date archived/stock date	December 2022
Cell line repository/bank	TMPi010-A (https://hpscreg.eu/cell-line/TMPi010-A)TMPi010-B (https://hpscreg.eu/cell-line/TMPi010-B)TMPi011-A (https://hpscreg.eu/cell-line/TMPi011-A)TMPi011-B (https://hpscreg.eu/cell-line/TMPi011-B)TMPi008-B (https://hpscreg.eu/cell-line/TMPi008-B)TMPi012-A (https://hpscreg.eu/cell-line/TMPi012-A)TMPi012-B (https://hpscreg.eu/cell-line/TMPi012-B)
Ethical approval	Cantonal Ethics Committee (BASEC-Nr.-2016–00101 & BASEC-Nr.-201700825)

1. Resource utility

Using iPSCs can be a powerful tool to model ADHD. One crucial advantage of using iPSCs is the conservation of the genetic information from patients. This allows us to replicate the genotype and phenotype of specific neural cell types in vitro and therefore, study the molecular mechanisms underlying this disorder.

2. Resource details

Attention-deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder typically characterized by impulsivity, hyperactivity and inattentiveness with a prevalence of 7.2% in children younger than 18 years old (Thomas et al., 2015). The etiology of ADHD is multifactorial, in which genetics and environmental factors play an important role. The heritability of ADHD is estimated at 74% suggesting a strong genetic component (Faraone and Larsson, 2019). In this paper, we discuss the quality control of seven cell lines (Table 1) originating from Peripheral blood mononuclear cells (PBMCs) from two ADHD patients and two healthy controls, reprogrammed by Sendai virus transduction.

Light microscopy imaging confirmed regular iPSC morphology, which were composed of small, rounded cells with high nuclei:cytoplasm ratio and grouped in colonies with well-defined borders (Fig. 1A). Immunocytochemistry assays indicated positive expression of the pluripotency markers TRA-1–60, OCT4, SOX2 and SSEA4 (Fig. 1A, scale bar: 200 μm). Minor or no Sendai virus (SeV) traces were detected in our cell lines and in the negative control (named as “iPSC control”) by RT-qPCR as opposed to the positive control as illustrated in Fig. 1B (P1 iPSC, which represented 100% of expression of SeV genome). All lines showed a minimal expression of SeV genome relative to the positive control (in a range of 0 % to 0.53%). Genomic integrity of iPSCs after reprogramming was confirmed by genotyping and Copy Number Variation (CNV) analysis (Fig. 1C depicts a normal karyotype for K015 c1; see supplementary material for the karyograms of all cell lines). Mycoplasma testing shows a defined band for the positive control column at 259 bp, which is not detected in the tested cell lines, confirming no Mycoplasma contamination. The negative control with a 481 bp band proves that the PCR was successful (Fig. 1D). Gene expression analysis revealed that all cell lines expressed the pluripotency genes *NANOG*, *OCT4*, *LIN28A* and *SOX2* as compared to a control iPSC line (a human iPSC CD34 + cord blood-derived iPSC generated by a three-plasmid, seven-factor EBNA-based episomal reprogramming system, which is commercially available at ThermoFisher Scientific under the catalog number A18945) (Burridge et al., 2011)(Fig. 1E). To determine the potential of the cells to differentiate into the three germ layers, embryoid bodies (EBs) were generated and their morphology was verified by light microscopy (Fig. 1A, scale bar: 200 μm). The presence of specific markers of ectoderm (*SOX2* and *PAX6*), mesoderm (*FLK1* and *ACTA2*) and endoderm (*AFP* and *SOX17*) layers confirmed their differentiation potential (Fig. 1F). The graphs show relative gene expression per gene normalized to reference genes.

3. Materials and methods

3.1. Recruitment of subjects

All subjects from this study (children and adolescents with or

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Light microscopy imaging	iPSC colonies showed regular morphology, with well-defined borders	Fig. 1 panel A
Phenotype	Qualitative proteomic analysis (Immunocytochemistry)	Positive expression of TRA-1–60, OCT4, SOX2 and SSEA4	Fig. 1 panel A
		Tested passage numbers: K013 c20: 12 K013 c39: 13 K015 c1: 11 K015 c9: 11 MR013 c13: 10 MR023 c17: 13 MR023 c22: 10	
	Quantitative transcriptomic analysis (RT-qPCR)	Positive expression of NANOG, OCT4, LIN28A, SOX2	Fig. 1 panel E
Genotype	Genetic integrity analysis in DNA from saliva and iPSCs through verification of CNVs	K013 c20: 46, XX (tested passage number: 13)K013 c39: 46, XX (tested passage number: 11)K015 c1: 46, XY (tested passage number: 11)K015 c9: 46, XY (tested passage number: 11)MR013 c13: 46, XY (tested passage number: 10)MR023 c17: 46, XY (tested passage number: 14)MR023 c22: 46, XY (tested passage number: 10)	Fig. 1 panel C and supplementary
Identity	Infinium Global Screening Array (Illumina)	N/A	N/A
	STR analysis	N/A	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	N/A	N/A
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Negative	Fig. 1 panel D
	Sendai Virus	Negligible	Fig. 1 panel B
Differentiation potential	Embryoid body formation	Successful EB generation and expression of ectodermal (SOX2 and PAX6), mesodermal (FLK1 and ACTA2) and endodermal (AFP and SOX17) markers	Fig. 1 panel A and F
List of recommended germ layer markers	Expression of these markers has to be demonstrated at mRNA (RT PCR) or protein (IF) levels, at least 2 markers need to be shown per germ layer	Ectoderm: PAX6 and SOX2; Endoderm: AFP and SOX17; Mesoderm: ACTA2 and FLK1	RT-qPCR of the specified markers using ACTB and GAPDH as housekeeping genes
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Genotyping from salivary DNA	DNA analysis	Not shown but available with author
	HLA tissue typing	N/A	N/A

without ADHD between 9 and 16 years old) were recruited by the Department of Child and Adolescent Psychiatry and Psychotherapy, University Hospital of Psychiatry Zurich, University of Zurich. Healthy controls and ADHD patients (who respond to Methylphenidate treatment) have undergone clinical and behavioural tests, as well as fulfil specific inclusion and exclusion criteria (see Supplementary Information).

3.2. Reprogramming

The four Yamanaka factors (Klf4-Oct3/4-Sox2 (KOS), cMyc and Klf4) were transduced into fresh Peripheral Blood Mononuclear Cells (PBMCs) of two ADHD patients and two healthy controls via Sendai virus (CytoTune™-iPS 2.0 kit from Invitrogen), in MOIs (Multiplicity of Infection) of 5, 5 and 3, respectively. Therefore, the number of PBMCs considered for transduction were 2.5 × 105. After transfection according to the manufacturers' instructions, PBMCs were centrifuged for 30 min at 1000 xg to increase reprogramming efficiency and thereafter, were seeded in a 12-well containing an additional 1 mL of StemPro-34 media (Gibco, 10639011) and incubated at 37°C and 5% CO2. On the following day, the cells were centrifuged at 200 xg for 10 min and the cells were resuspended in StemPro-34 media containing 10 μL/mL of L-glutamine (Gibco), IL-3 (20 ng/mL), IL-6 (20 ng/mL), FLT3 (100 ng/mL) and SCF (100 ng/mL) (Peprotech) and cultured at 37°C and 5% CO2. Three days after the transduction, reprogramming PBMCs were plated onto 6-well plates coated with Matrigel (Corning) (diluted 1:100 in DMEM/F12 media (Gibco, 11320033)) and in Essential 8 Medium (E8) (Gibco, A1517001) at 37°C and 5% CO2. Cells were monitored daily until the formation of colonies. After clone isolation through manually picking of colonies, the iPSCs were cultivated in 6-well plates coated with Vitronectin 5 μg/mL (diluted in PBS 1x) in E8 media with Rock inhibitor 5 μM, which was gradually transitioned to Essential 8 Flex media (Gibco, A2858501) when they reached passage 10. Colonies were passaged at a 1:3 ratio using Versene (Gibco) whenever 80–90% confluence was reached. Quality control steps were performed when iPSCs reached at least passage 10.

3.3. Immunocytochemistry

Using the Pluripotent Stem Cell 4-marker Immunocytochemistry Kit (Invitrogen), the iPSCs were stained with specific antibodies to visualise the protein expression of 4 pluripotent markers: OCT4, SSEA4, TRA-1–60 and SOX2, according to the manufacturer's instructions (Table 2). All reagents were provided with the kit. The iPSCs were cultured on 96-

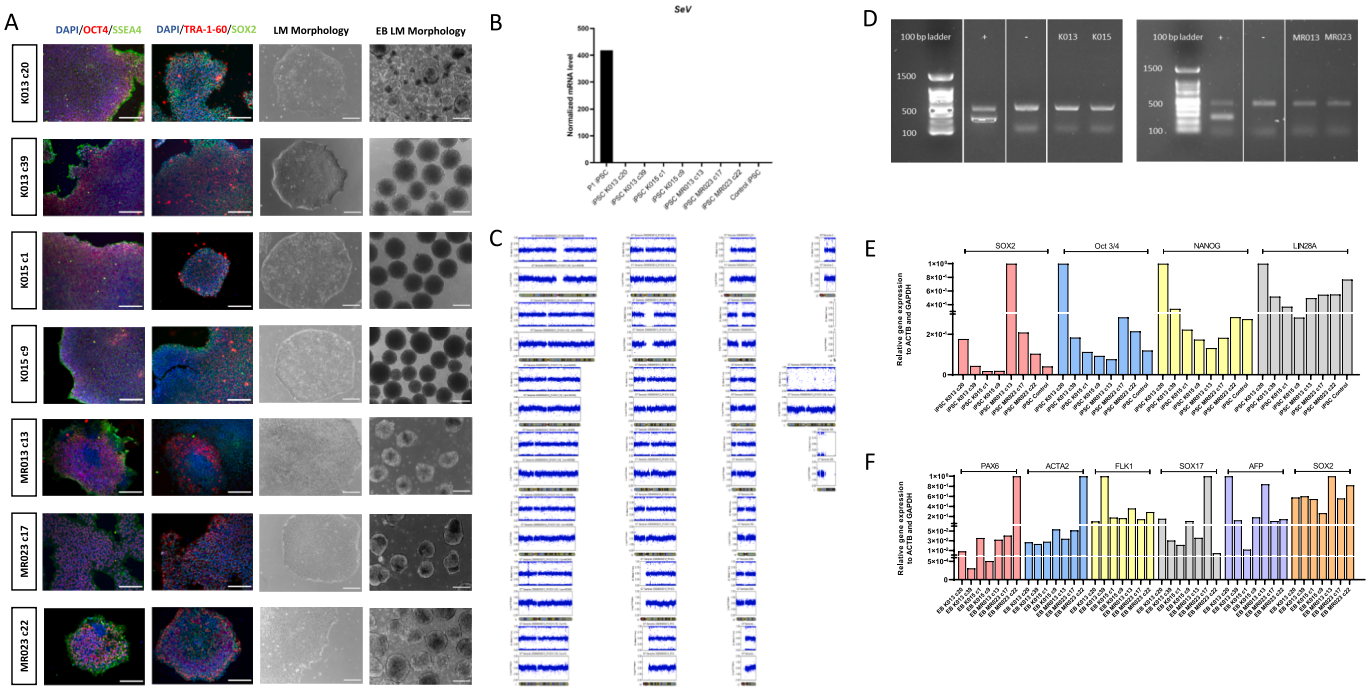

Fig. 1. .

Table 2
Reagents details.

	Antibodies used for immunocytochemistry			
	Antibody	Dilution	Company Cat #	RRID
Pluripotency Markers	Rabbit anti-OCT4	1:200	Thermo Fisher Scientific Cat# A24867	AB_2650999
Pluripotency Markers	Mouse IgG3 anti-SSEA4	1:100	Thermo Fisher Scientific Cat# A24866	AB_2651001
Pluripotency Markers	Rat anti-SOX2	1:100	Thermo Fisher Scientific Cat# A24759	AB_2651000
Pluripotency Markers	Mouse IgM anti-TRA-1–60	1:100	Thermo Fisher Scientific Cat# A24868	AB_2651002
Secondary Antibodies	Alexa Fluor™ 555 donkey anti-rabbit	1:250	Thermo Fisher Scientific Cat# A24869	AB_2651006
Secondary Antibodies	Alexa Fluor™ 488 goat anti-mouse IgG3	1:250	Thermo Fisher Scientific Cat# A24877	AB_2651008
Secondary Antibodies	Alexa Fluor™ 488 donkey anti-rat	1:250	Thermo Fisher Scientific Cat# A24876	AB_2651007
Secondary Antibodies	Alexa Fluor™ 555 goat anti-mouse IgM	1:250	Thermo Fisher Scientific Cat# A24871	AB_2651009
	Primers			
	Target	Size of band	Forward/Reverse primer (5′-3′)	
Pluripotency Markers (qPCR)	LIN28A	126 bp	According to manufacturer Qiagen 249,900 (QT00014938)	
Pluripotency Markers (qPCR)	NANOG	90 bp	According to manufacturer Qiagen 249,900 (QT01025850)	
Pluripotency Markers (qPCR)	OCT4	77 bp	According to manufacturer Qiagen 249,900 (QT00210840)	
Pluripotency Markers (qPCR)	SeV	181 bp	F: GGATCACTAGGTGATATCGAGC R: ACCAGACAAGAGTTTAAGAGTATGTATC	
Ectodermal Markers (qPCR)	PAX6	113 bp	F: AACGATAACATACCAAGCGTGT R: GGTCTGCCCGTTCAACATC	
Ectodermal Markers (qPCR)	SOX2	64 bp	According to manufacturer Qiagen 249,900 (QT00237601)	
Mesodermal Markers (qPCR)	ACTA2	83 bp	According to manufacturer Qiagen 249,900 (QT00088102)	
Mesodermal Markers (qPCR)	FLK1	131 bp	F: TGATCGGAAATGACACTGGA R: CACGACTCCATGTTGGTCAC	
Endodermal Markers (qPCR)	SOX17	119 bp	According to manufacturer Qiagen 249,900 (QT00204099)	
Endodermal Markers (qPCR)	AFP	136 bp	F: AAATGCGTTTCTCGTTGCTT R: GCCACAGGCCAATAGTTTGT	
House-Keeping Genes (qPCR)	ACTB	146 bp	According to manufacturer Qiagen 249,900 (QT00095431)	
House-Keeping Genes (qPCR)	GAPDH	95 bp	According to manufacturer Qiagen 249,900 (QT00079247)	
House-Keeping Genes (qPCR)	HMBS	107 bp	According to manufacturer Qiagen 249,900 (QT00014462)	

wells, and media was removed prior to fixation. For fixation of the iPSCs, cells were incubated with Fixative Solution for 15 min at room temperature (RT). Following, the cells were washed 3 times (5 min each) with Wash Buffer 1x. Next, cells were incubated for 15 min with Permeabilization Solution followed by a 30-minute incubation with Blocking Solution. Primary antibodies were added to the wells, which were incubated overnight at 4°C. On the following day, primary antibodies were removed for a second wash round with Wash Buffer 1x for 3 times (5 min each). Secondary antibodies were incubated for 30 min at RT, in the dark. The iPSCs were washed two more times with Wash Buffer 1x. NucBlueTM Fixed Cell Stain (1 drop/well in a five-minute incubation) was used to stain nuclear DNA, followed by one final wash with Wash Buffer 1x for 5 min. Finally, Dako Fluorescence Mounting Media (Agilent) was added to the wells (1 drop/well in a five-minute incubation).

3.4. Real-time quantitative PCR (RT-qPCR)

For the generated iPSCs and the commercial human cord blood-derived iPSC used as control (Burridge et al., 2011), reprogrammed through the transfection of seven-factor EBNA-based episomal vectors), RT-qPCR analysis was done to establish gene expression of different pluripotency genes: *LIN28A*, *NANOG*, *OCT3/4* and *SOX2* (detailed method see (Yde Ohki et al., 2021)). For Embryoid Bodies (EBs), the expression of *AFP*, *SOX17*, *ACTA2*, *FLK1*, *PAX6* and *SOX2* was analyzed. BioRad's iScriptTM cDNA Synthesis Kit was used to reverse-transcribe 500 ng RNA into cDNA from iPSCs using the RNeasy® Plus Mini kit (Qiagen). Complementary DNA (cDNA) production and amplification of genes of interest (GOIs) and reference genes (RGs) were performed on the CFX384 thermal cycler. Cycling conditions consisted of Priming (for 5 min at 25°C), reverse transcription (for 20 min at 46°C) and enzyme inactivation (for 1 min at 95°C). Amplification reactions were performed by using the QuantiNova® SYBR® Green PCR kit (Qiagen). Cycling conditions included denaturation for 5 s at 95°C, followed by 40 cycles of combined annealing and extensions (95°C for 10 s and 60°C for 30 s). These conditions were applied to most primers, which had 60°C as annealing temperature, except for *FLK* (63.3°C) and *AFP* (58.8°C). LinReg software was used to obtain amplification efficiencies per gene while the Biogazelle qBasePLUS2 software (version 2.3) was used to normalize GOIs' mRNA levels in relation to housekeeping genes (*ACTB*, *HMBS* and *GAPDH*) (see primers in Table 2).

3.5. Detection of Sendai virus traces through RT-qPCR

The presence of possible Sendai virus traces was tested by SeV-specific primers in RT-qPCR reactions (Table 2), as described in the section above (detailed method see (Yde Ohki et al., 2021)). A positive control (an iPSC line at passage 1) and negative control (the aforementioned episomal control iPSC) (Burridge et al., 2011) were added.

3.6. Mycoplasma testing

Supernatants were collected from iPSC cultures above passage 10 and used for analysis using the LookOut Mycoplasma PCR Detection Kit (Sigma-Aldrich) following the manufacturer's instructions. To amplify the mycoplasma DNA, if present, C1000TM/CFX96TM Thermal Cycler (BioRad) was used, and amplicons were loaded on a 1.2% agarose gel containing HDGreen Plus DNA Stain (INTAS, Germany) and run at 100 V for 30 min. Bands were visualized with the software through conventional PCR followed by gel electrophoresis.

3.7. Embryoid Bodies (EBs) generation from iPSCs

EBs were generated in Aggrewells using an adapted version of Lin, Y. and Chen, G. (2014) (Lin and Chen, 2014). After 48 h in culture at 37°C and 5% CO2, RNA extraction was performed and 1 μg was used as a template for cDNA production and subsequent RT-qPCR experiments, as described above.

3.8. Genotyping analysis

Genotyping analysis was performed by extracting DNA using the GeneFixTM Saliva-Prep DNA kit (Isohelix) and the DNeasy® Blood & Tissue kit (Qiagen) from iPSCs and Saliva, respectively. The protocol followed was provided by the respective manufacturers. Genotyping was done with Infinium Global Screening Array (GSA, Illumina) of the sample prior to analysis and comparison between the genotypes using the Genome Viewer function from the GenomeStudio software (version 2.0).

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2023.103084.

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