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Lab Resource: Multiple Cell Lines

Generation of four iPSC lines from peripheral blood mononuclear cells (PBMCs) of an attention deficit hyperactivity disorder (ADHD) individual and a healthy sibling in an Australia-Caucasian family

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ABSTRACT

Peripheral blood mononuclear cells were donated by a male teenager with clinically diagnosed attention deficit hyperactivity disorder (ADHD) under the Diagnostic and Statistical Manual of Mental Disorders IV criteria and his unaffected male sibling. Induced pluripotent stem cells were developed using integration-free Sendai Reprogramming factors containing OCT4, SOX2, KLF4, and c-MYC. All four iPSC lines displayed pluripotent cell morphology, pluripotency-associated factors at the DNA and protein level, alkaline phosphatase enzymatic activity and a male karyotype of 46, XY. All lines had capacity for *in vitro* differentiation into all the three germ layers. All were negative for Mycoplasma.

Resource table

Unique stem cell lines identifier	1. *MICCNi001-A* 2. *MICCNi001-B* 3. *MICCNi002-A* 4. *MICCNi002-B*
Alternative names of stem cell lines	*N/A*
Institution	*Monash Institute of Cognitive and Clinical Neurosciences, Melbourne, Australia*
Contact information of distributor	*Dr Janette Tong (janette.tong@monash.edu)*
Type of cell lines	*iPSC*
Origin	*Human*
Cell source	*Peripheral blood mononuclear cells*
Clonality	*Clonal*
Method of reprogramming	*CytoTune®-iPS 2.0 Sendai Reprogramming Kit*
Multiline rationale	*ADHD-affected and unaffected male pair*
Gene modification	*NO*
Type of modification	*N/A*
Associated disease	*Attention deficit hyperactivity disorder*
Gene/locus	*N/A*
Method of modification	*N/A*
Name of transgene or resistance	*N/A*
Inducible/constitutive system	*N/A*
Date archived/stock date	*August 7, 2018*
Cell line repository/bank	*Details for all cell lines can be assessed fromhttps://hpscreg.eu/cell-line/MICCNi001-A; https://hpscreg.eu/cell-line/MICCNi001-B; https://hpscreg.eu/cell-line/MICCNi002-Aandhttps://hpscreg.eu/cell-line/MICCNi002-B.*
Ethical approval	*Monash University Human Research Ethics Committee, CF15/2566 – 2015001048*

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1. Resource utility

The pathophysiology of attention deficit hyperactivity disorder (ADHD) is poorly understood due to a lack of cellular models that faithfully represent the clinical ADHD features, and a limited availability of live tissues for long-term mechanistic studies. We generated iPSCs from a patient and a unaffected sibling to understand the pathophysiology of ADHD.

2. Resource details

A blood sample was donated from a pair of dizygotic twin brothers; one brother met the Diagnostic and Statistical Manual of Mental Disorders IV (*DSM-IV*) criteria (Dupaul et al., 2001) for ADHD, his male sibling was unaffected. Both individuals completed the Conners' Parent Rating Scale-Revised: Long Form (CPRS-R:L) which is designed to assess symptoms (inattention, impulsivity, hyperactivity) of ADHD (Conners et al., 1998). For the child's gender and age, the affected sibling scored above the 98th percentile on the Impulsivity subscale and above the 95th percentile on the Hyperactivity, ADHD Index and *DSM-IV* Inattentive subscales of CPRS-R:L, which is consistent with the diagnostic interview conducted by an experienced psychiatrist. The unaffected sibling scored at or below average *T*-Scores and percentiles across all CPRS-R:L subscales. The affected sibling was managed by Ritalin and fluoxetine at the time of testing and sample collection. The study was approved by the Monash University Human Research Ethics Committee Australia under the approval number, CF15/2566 – 2015001048.

Four induced pluripotent stem cell (iPSC) lines (two from the affected sibling- MICCNi002-A and -B; two from the unaffected sibling - MICCNi001-A and -B) were generated from peripheral blood mononuclear cells (PBMCs) by reprograming with integration-free Sendai viral vectors expressing *KLF4*, *OCT3/4*, *c-MYC* and *SOX2*. All iPSC colonies showed a round compact shape with clear peripheral outline, which is typical colony morphology (Fig. 1A). RT-PCR analyses of the viral genome and transgenes confirmed that our iPSC lines were Sendai-negative and vector-free after 10 passages (Fig. 1B). Quantitative RT-PCR (qRT-PCR) of iPSC lines at passage 10 showed comparable fold changes in expression of pluripotency-related genes *POU5F1* and *SOX2* in all lines when compared to the positive control, an established iPSC line at passage number 33 (Fig. 1C). Immunocytochemistry analyses of MICCNi001-A and -B and MICCNi002-A and -B at passage number 12 showed strong expression of pluripotency markers TRA 1–60, OCT4, and NANOG, and were positive for alkaline phosphatase enzymatic activity (Fig. 1D and E). Mycoplasma testing of all lines at passage 15 was negative by luminescence (Supp. File 1). G-band analyses at passage 10 exhibited normal male karyotypes of 46, XY for MICCNi002-A (Fig. 1F) and others (Suppl. File 1). Our iPSC lines had adequate potential for *in vitro* differentiation into the three germ layers *via* embryoid bodies using a conventional method (Fig. 1G). Subsequently, the generated embryoid bodies were tested for expression of > 80 differentiation-associated markers for all three germ layers as well as key pluripotency genes using the hPSC Scorecard assay. All *in vitro* differentiated iPSC lines showed reduced expression of self-renewal genes and upregulation of markers for mesendoderm, ectoderm, mesoderm and endoderm (Fig. 1H). Lastly, MICCNi001-A, MICCNi001-B, MICCNi002-A and MICCNi002-B lines were authenticated with 100%

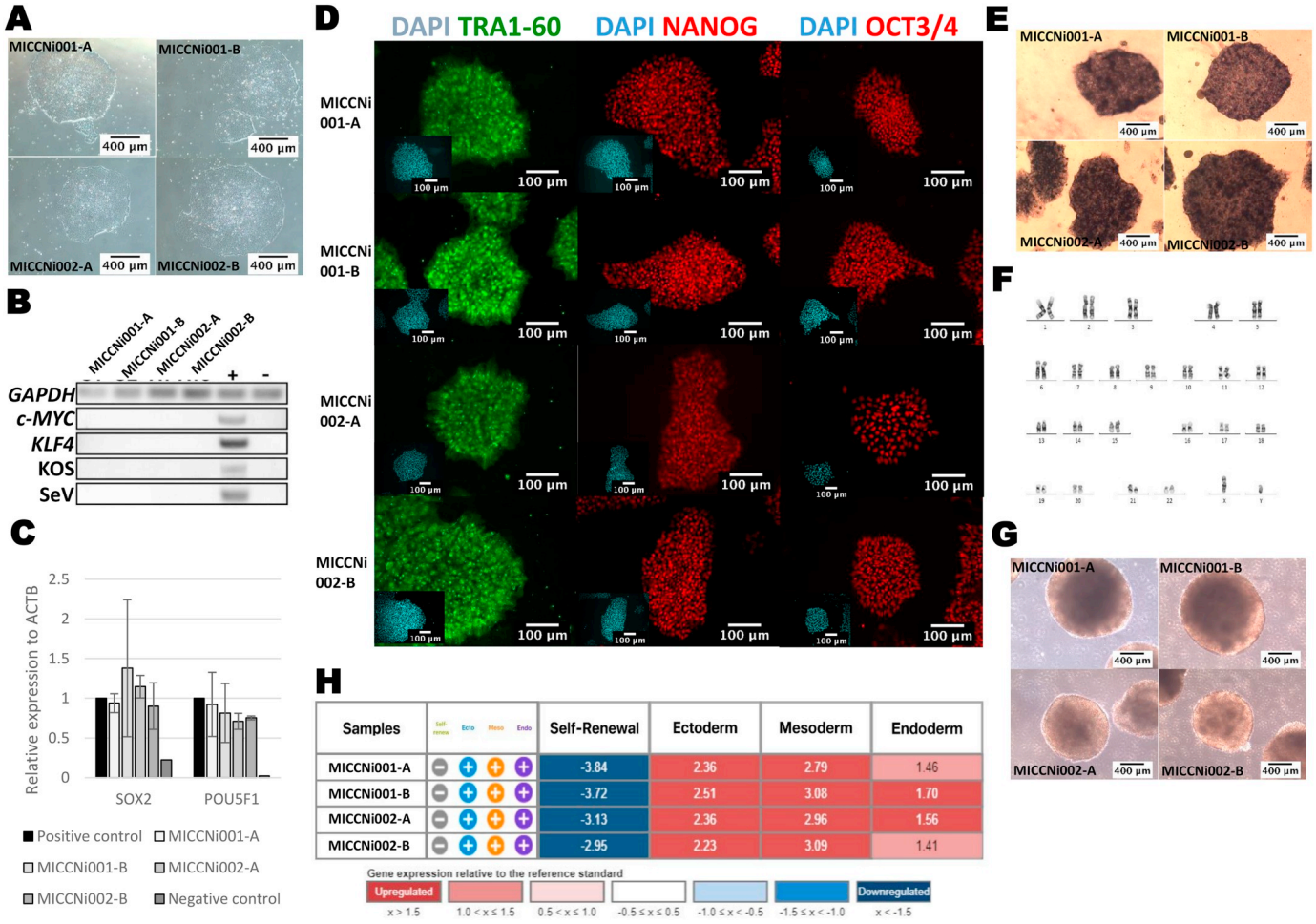

Fig. 1. Characterisation and validation of MICCNi001-A, MICCNi001-B, MICCNi002-A and MICCNi002-B.

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
MICCNi001-A	MICCNi001-A	Male	16	Caucasian	N/A	Healthy
MICCNi001-B	MICCNi001-B	Male	16	Caucasian	N/A	Healthy
MICCNi002-A	MICCNi002-A	Male	16	Caucasian	N/A	Attention Deficit Hyperactivity Disorder
MICCNi002-B	MICCNi002-B	Male	16	Caucasian	N/A	Attention Deficit Hyperactivity Disorder

concordance with parental PBMCs using Short Tandem Repeat profiling (available with the authors).

3. Materials and methods

3.1. Human sample

Human peripheral blood mononuclear cells (PBMCs) were isolated from 30 mL blood in Leucosep™ Tubes (Greiner Bio-One) filled with 15 mL Ficoll-Paque Plus™ (GE Healthcare). Within 1 h of collection, the blood sample was centrifuged at 800 $\times g$ for 15 min at room temperature (RT), washed three times with PBS and centrifuged at 250 $\times g$ for 10 min at RT. 1×10^6 cells were frozen in 20% Dimethyl sulfoxide and 80% Fetal Bovine Serum (Bovogen) until Sendai transduction.

3.2. PBMC reprogramming, iPSC generation and culture

After recovery from cryopreservation PBMCs (5×10^5 cells per well of a 24-well plate 4 days prior to transduction) were cultured in PBMC medium until transduction with Sendai virus particles containing *OCT*, *SOX2*, *KLF4*, and *c-MYC* using CytoTune®-iPS 2.0 Sendai Reprogramming Kit (Life Technologies) according to manufacturer's instruction. The PBMCs were initially seeded in PBMC medium and half of the medium was replaced with fresh medium until the transduction, where the reprogrammed cells were plated on irradiated Mouse Embryonic Fibroblasts (MEFs) in StemPro®-34 medium without cytokines the subsequent day (Nefzger et al., 2014). The cells were grown on MEFs in iPSC medium for 20 days with an addition of fresh MEF culture dishes every 7 days to allow colony formation. Each colony was manually picked, transitioned in culture vessels coated with Vitronectin (Thermofisher) and cultured in E8 medium (Invitrogen). The reprogrammed cells were passaged when confluent, trypsinized by 0.5 µM EDTA (Invitrogen) in 1/20 ratio every 10 days, and cultured on vitronectin-coated plates in E8 with ROCK inhibitor (Merck-Millipore) for 24 h after the passaging. The ROCK-inhibitor was removed, and the media was replaced every day during the expansion. Both PBMCs and iPSCs were cultured at 37 °C with 5% CO_2.

3.3. Immunocytochemistry and alkaline phosphatase staining of iPSC

Pluripotency of iPSCs were examined by immunocytochemistry analyses with TRA 1–60, OCT3/4, and NANOG antibodies. Cells were seeded from one well of a 6-well culture plate on vitronectin-coated eight-chamber culture slide (Falcon, USA) in the iPSC media with ROCK inhibitor for 6 days. Cells were fixed in 4% formaldehyde solution (Merck) for 15 min at RT, washed with PBS and incubated with 0.1% Triton X 100 for 30 min. Cells were subsequently washed with PBS and incubated with 5% donkey serum (Merck-Millipore) in PBS with 0.1% Tween 20 (Thermofisher), PBST, for 30 min in RT. Cells were incubated with primary antibodies for TRA 1–60, OCT3/4, or NANOG diluted in PBST (Table 3) for 24 h at 4 °C. Cells were then washed three times with PBS and incubated with Secondary Alexa Fluor antibodies diluted in PBST (Table 3) for 16 h at 4 °C. Lastly, cells were washed 3 × PBS and fixed with Fluroshield™ with DAPI (Sigma-Aldrich). Images were acquired using IX71 inverted microscope (Olympus) and processed in ImageJ software. Alkaline phosphatase staining was performed using Vector® Black Alkaline Phosphatase Substrate Kit II as per manufacturer's instruction (Vector Laboratories, USA) Tables 1 and 2.

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Bright-field microscopy	Normal with colony borders	Fig. 1A
Phenotype	Qualitative analysis: immunocytochemistry	Positive expression of pluripotency markers (TRA 1–60, OCT4 and NANOG)	Fig. 1D
	Alkaline phosphatase enzymatic activity	Positive for alkaline phosphatase activity	Fig. 1E
	Quantitative analysis: qRT-PCR	Positive for *POU5F1* and *SOX2*.	Fig. 1C
Genotype	Karyotype (G-banding) and resolution	46,XY Resolution 300–400	Fig. 1F, Supplementary file 1A
Identity	STR analysis report	Performed DNA Profiling of 15 loci with 100% concordance with parental PBMCs	Available with the authors
Mutation analysis (If Applicable)	Sequencing	N/A	N/A
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by luminescence using MycoAlert™ mycoplasma detection kit (Lonza) - Negative	Supplementary file 1B
Differentiation potential	Embryoid body formation and Scorecard	Differentiation markers such as PAX6 for ectoderm, ODAM for mesoderm and AFP for endoderm are markedly upregulated from the undifferentiated reference standard of Scorecard	Fig. 1G and H
Donor screening (optional)	N/A	N/A	N/A
Genotype additional info (Optional)	N/A	N/A	N/A
Genotype additional info (Optional)	N/A	N/A	N/A

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Primary antibodies for TRA 1–60	Monoclonal anti-mouse TRA 1–60	1:300	560,071 (BD Biosciences) and AB_1645604
Primary antibodies for NANOG	Polyclonal anti-rabbit NANOG	1:100	Ab21624 (Abcam) and AB_446437
Primary antibodies for OCT3/4	Monoclonal anti-mouse OCT3/4	1:100	SC-5279 (Santa Cruz) and AB_628051
Secondary antibodies for TRA 1–60	Goat anti-mouse IgM, Alexa Fluor 488	1:400	A21042 (Life Technologies) and AB_141357
Secondary antibodies for NANOG	Goat anti-rabbit IgG, Alexa Fluor 555	1:400	A21428 (Life Technologies) and AB_141784
Secondary antibodies for OCT3/4	Goat anti-mouse IgG 2b, Alexa Fluor 555	1:400	A21147 (Life Technologies) and AB_2535783

Primers			
	Target	Expected size	Forward/Reverse primer (5'-3')
Episomal Plasmids (RT-PCR)	KOS	528 bp	ATGCACCGCTACGACGTGAGCGC/ACCTTGACAATCCTAATGTGG
Episomal Plasmids (RT-PCR)	*c-MYC*	532 bp	TAACTGACTAGCAGGCTTGTCG/TCCACATACAGTCCTGGATGATGATG
Episomal Plasmids (RT-PCR)	*KLF4*	410 bp	TTCCTGCATGGCAGAGGAGCCC/AATGTATCGAAGGTGCTCAA
Episomal Plasmids (RT-PCR)	*SeV*	181 bp	GGATCACTAGGTGATATCGAGC/ACCAGACAAGAGTTTAAGAGATATGTATC
House-Keeping Genes (RT-PCR)	*GAPDH*	157 bp	TGAAGGTCGGAGTCAACGGA/CCAATTGATGACAAGCTTCCCG
Endogenous *POU5F1* (qRT-PCR)	*POU5F1*	114 bp	GCTGGAGCAAAACCCGGAGG/TCGGCCTGTGTATATCCCAGGGTG
Endogenous *SOX2* (qRT-PCR)	*SOX2*	122 bp	GAGAAGTTTGAGCCCCAGG/AGAGGCAAACTGGAATCAGG
House-Keeping genes (qRT-PCR)	*ACTB*	172 bp	ACCACACCTTCTACAATGAGC/GCGTACAGGGATAGCACAG

3.4. Karyotyping

G-banding of iPSCs was carried out at the Monash Pathology Services (Monash Medical Centre). A total of 15 cells were analysed; 5 banded cells were analysed for metaphases spread and 10 cells were examined for chromosome analysis.

Reverse transcription polymerase chain reaction (RT-PCR) of Sendai vectors and pluripotency markers.

For all RT-PCR applications, total RNA was isolated using TRIzol (Life technologies) according to manufacturer's instruction. The optical density at 260/280 of all RNA preparations ranged from 2 to 2.1 using Nanodrop™ Spectrophotometer (Thermofisher) prior to reverse transcription reaction. 1 μg RNA was converted into cDNA by SuperScript IV Reverse Transcriptase kit (Thermofisher) and 2 μL cDNA was used for polymerase chain reactions with primers (Table 3) by T-100™ thermal cycler (BioRad). Sendai vector transgenes *KLF4*, *OCT3/4*, *c-MYC* and *SOX2* were amplified with HOT FIREPol® DNA Polymerase (Solis BioDyne) and primers (Table 3) by T-100™ thermal cycler (BioRad). The following cycling conditions are used: initial denaturation at 95 °C for 3 min, followed by 35 cycles of amplification (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s) and a final extension at 72 °C for 5 min. *POU5F1* and *SOX2* expression was assessed in triplicate using the SYBR Green master mix (Roche) on LightCycler 480 II instrument (Roche), using *B2M* and *ACTB* housekeeper genes (Table 3). The PCR efficiency of *POU5F1*, *SOX2* and *ACTB* was determined as described previously and analysed using the ΔΔCt method (Schmittgen and Livak, 2008). Data are shown as fold changes in gene expression relative to controls, after normalization with housekeeper gene *ACTB*. Data represent one of two independent experiments. Error bars represent standard deviation of 3 biological replicates.

3.5. Embryoid bodies (EB) formation and ScoreCard

The differentiation capacity of iPSC lines was analysed by the formation of EB as per manufacturer's instructions of the ScoreCard assay (Thermofisher). Briefly, iPSCs were dissociated and cultured non-adherently in EB differentiation medium for 7 days. The total RNA of cells was extracted and converted into cDNA using the aforementioned methods. Quantitative analyses of cDNA samples were performed in Applied Biosystems 7900HT Fast Real-Time PCR System using the TaqMan hPSC Scorecard Assay (Thermofisher).

3.6. Short tandem repeat analysis

All genomic DNA was extracted from PBMCs and iPSCs using PureLink genomic DNA Mini kit (Thermofisher) according to manufacturer's instruction, and were analysed at the Medical Genomics Facility (MHTP, Melbourne), where 16 loci were investigated by PowerPlex HS16 System kit (Promega).

3.7. Mycoplasma testing

The mycoplasma testing was carried out using Mycoalert™ mycoplasma detection kit (Lonza) following manufacturer's instruction.

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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.2018.11.014.

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