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 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 OCT, SOX2, KLF4, and c-MYC. All four iPSC lines displayed pluripotent cell morphology, pluripotency-associated factors at protein level, alkaline phosphatase enzymatic activity, male karyotype of 46, XY, and *in vitro* differentiation capacity into all the three germ layers and negative for Mycoplasma.

Introduction

The pathophysiology of Attention Deficit Hyperactivity Disorder (ADHD) is poorly understood due to the lack of cellular models that faithfully represent the clinical ADHD features, and limited live tissues

to expand for long-term mechanistic studies. We generated iPSCs from an ADHD patient and a unaffected sibling to understand the pathophysiology.

Results

A blood sample was donated from a pair of dizygotic twin brothers with one brother meeting the Diagnostic and Statistical Manual of Mental Disorders IV (*DSM-IV*) criteria (DuPaul et al., 2001) for ADHD and his male sibling being 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 is 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*. 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) the subsequent day (Nefzger et al., 2014). The cells were grown on MEFs for 20 days with an addition of fresh MEF culture dishes every 7 days to allow colony formation, and large colonies were dissected and transferred to vitronectin-coated flasks. 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). 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, NANOG and KLF4, as well as positive for alkaline phosphatase enzymatic activity (Fig. 1C and 1D). 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. 1E) and others (Suppl. File 1). Our iPSC lines have adequate potential for *in vitro* differentiation into the three germ layers via embryoid bodies using a conventional method (Thermofisher, 2013) (Fig. 1F). 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 of mesendoderm, ectoderm, mesoderm and endoderm (Fig 1G). Lastly, MICCNi001-A, MICCNi001-B, MICCNi002-A and MICCNi002-B lines were authenticated with 100% concordance with its parental PBMCs using Short Tandem Repeat profiling (Supp. File 2).

Materials and Methods

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 hour of collection, the blood sample was centrifuged at 800 × *g* for 15 min at room temperature (RT), washed three times with PBS and centrifuged at 250 × *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.

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 (Thermofisher, 2016) until transduction with Sendai virus particles containing *OCT*, *SOX2*, *KLF4*, and *c-MYC* using CytoTune®-iPS 2.0 Sendai Reprogramming Kit (Life Technologies). Transduced cells were cultured on growth inactivated/irradiated mouse embryonic fibroblasts (MEFs) feeder-cells for 7 days in StemPro®-34 medium without cytokines. Thereafter cells were fed with fresh iPSC medium (Thermofisher, 2016) and monitored daily until large colonies were formed within a two week period. Each colony was manually picked, transitioned in culture vessels coated with Vitronectin (Thermofisher) and expanded in E8 medium (Invitrogen).

3. Immunocytochemistry and Alkaline Phosphatase staining of iPSC

Pluripotency of iPSCs cultured on Vitronectin-coated plates were examined by immunocytochemistry analyses with TRA 1-60, OCT3/4, NANOG or KLF4 antibodies. Cells were seeded at 1×10^5 cells on an eight-chamber, polystyrene vessel tissue culture treated glass slide (Falcon, USA) 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 antibodies for TRA 1-60, OCT3/4, NANOG or KLF4 (Table 3) for 24 hours at 4°C. Cells were then washed three times with PBS and incubated with Secondary Alexa Fluor antibodies (Table 3) for 2 hours. 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 manufactures instructions (Vector Laboratories, USA).

4. Karyotyping

G-banding of iPSCs was carried out at the Monash Pathology Services (Monash Medical Centre).

5. Embryoid bodies (EB) formation and ScoreCard

The differentiation capacity of iPSC lines was analyzed by the formation of EB as per manufacturer's instructions of the ScoreCard assay (Thermofisher). Briefly, iPSCs were dissociated and culture non-adherently in EB differentiation medium for 7 days. Total RNA of cells were extracted using the RNeasy micro kit (Qiagen) and reverse transcribed into cDNA using the SuperScript III cDNA synthesis kit (Invitrogen). Quantitative analyses of cDNA samples were performed using the TaqMan hPSC Scorecard Assay (Thermofisher).

6. Short Tandem Repeat analysis

DNA extracted from PBMCs and iPSCs were sent to the Medical Genomics Facility (MHTP, Melbourne) for STR analysis, where 16 loci were investigated by PowerPlex HS16 System kit (Promega).

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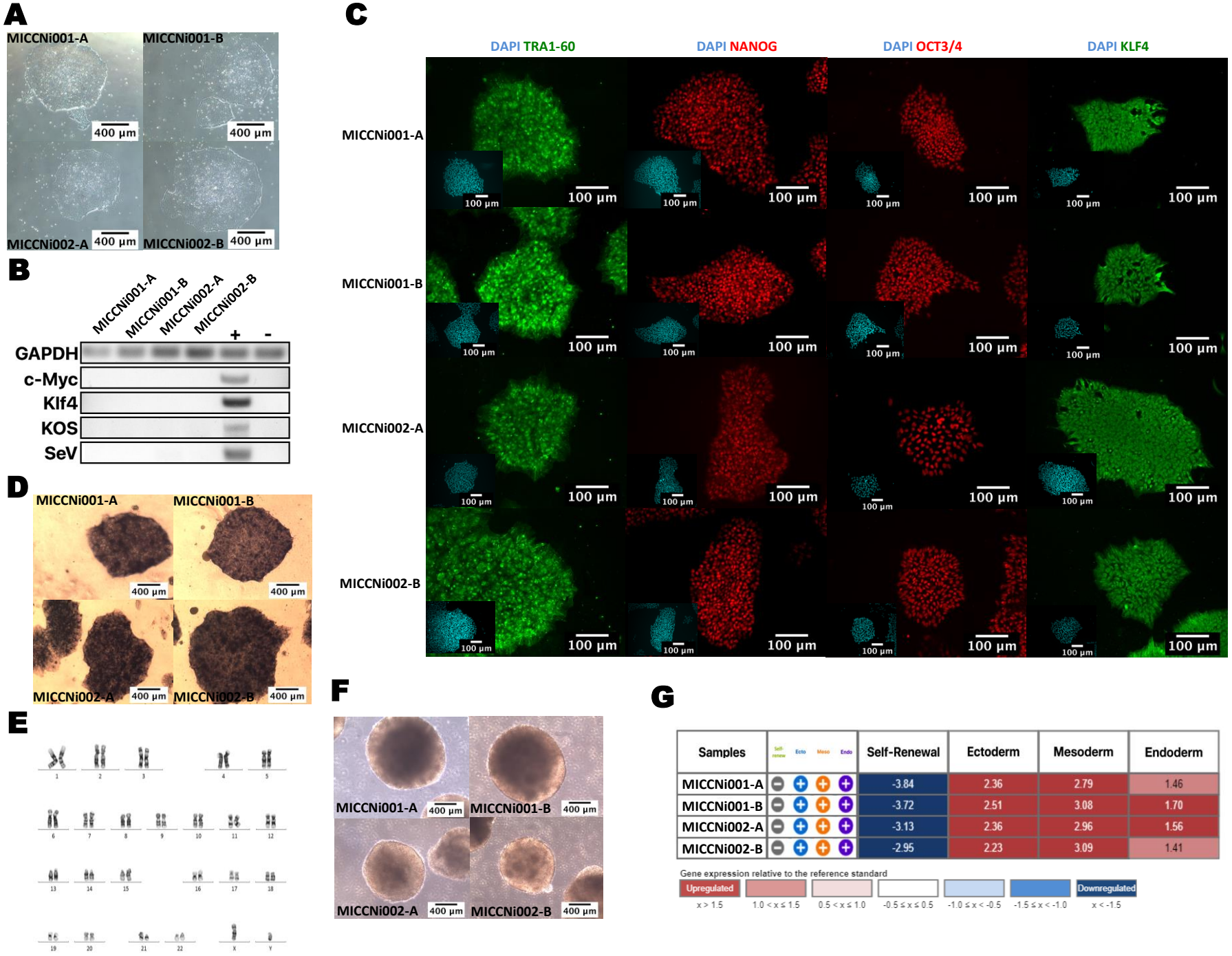
A
MICCNi001-A
MICCNi001-B
MICCNi002-A
MICCNi002-B
400 μm
B
MICCNi001-A
MICCNi001-B
MICCNi002-A
MICCNi002-B
+
-
GAPDH
c-Myc
Klf4
KOS
SeV
C
DAPI TRA1-60
DAPI NANOG
DAPI OCT3/4
DAPI KLF4
MICCNi001-A
MICCNi001-B
MICCNi002-A
MICCNi002-B
100 μm
D
MICCNi001-A
MICCNi001-B
MICCNi002-A
MICCNi002-B
400 μm
E
F
MICCNi001-A
MICCNi001-B
MICCNi002-A
MICCNi002-B
400 μm
G
Samples
Self-renew
Ecto
Meso
Endo
Self-Renewal
Ectoderm
Mesoderm
Endoderm
MICCNi001-A
-3.84
2.36
2.79
1.46
MICCNi001-B
-3.72
2.51
3.08
1.70
MICCNi002-A
-3.13
2.36
2.96
1.56
MICCNi002-B
-2.95
2.23
3.09
1.41
Gene expression relative to the reference standard
Upregulated
Downregulated
x > 1.5
1.0 < x ≤ 1.5
0.5 < x ≤ 1.0
-0.5 ≤ x ≤ 0.5
-1.0 ≤ x < -0.5
-1.5 ≤ x < -1.0
x < -1.5

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

Table 2: Characterization and validation

Classification	Test	Result	Data
Morphology	Bright-field microscopy	Normal with colony borders	Figure 1A
Phenotype	Immunocytochemistry and alkaline phosphatase enzymatic activity	Positive expression of pluripotency markers TRA 1-60, OCT4, NANOG and KLF4	Figure 1C Figure 1D
	RT-qPCR	No detected expression of Sendai virus genome (SeV) and the transgenes in the viral constructs - *KLF4*, *OCT3/4*, *c-MYC* and *SOX2*.	Figure 1B
Genotype	Karyotype (G-banding) and resolution	46,XY Resolution 300-400	Figure 1E, Supplementary file 1A
Identity	STR analysis report	Performed DNA Profiling of 15 loci with 100 % concordance with its parental PBMCs	submitted in archive with journal (uploaded as "STR analysis")
Mutation analysis (IF APPLICABLE)	N/A	N/A	N/A
Mutation analysis (IF APPLICABLE) **Microbiology and virology**	N/A	N/A	N/A
	Mycoplasma	Mycoplasma testing by luminescence using MycoAlert™ mycoplasma detection kit (Lonza) - Negative	Supplementary file 1B
Differentiation potential	Embryoid body formation and Scorecard	Pluripotent markers such as PAX6 for ectoderm, ODAM for mesoderm and AFP for endoderm are markedly	Figure 1F - bright-field showing embryoid bodies prior to Scorecard assay

		different from the undifferentiated reference standard of Scorecard	Figure 1G – hPSC Scorecard results
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	560071 (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
Primary antibodies for KLF4	Polyclonal anti-rabbit KLF4	1:100	SC-20691 (Santa Cruz) and AB_669567
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
Secondary antibodies for KLF4	Goat anti-rabbit IgG, Alexa Fluor 488	1:400	A11008 (Life Technologies) and AB_143165
Primers			
	Target	**Forward/Reverse primer (5′-3′)**	
Episomal Plasmids (RT-PCR)	*KOS*	ATGCACCGCTACGACGTGAGCGC/ ACCTTGACAATCCTAATGTGG	
Episomal Plasmids (RT-PCR)	*c-MYC*	TAACTGACTAGCAGGCTTGTCG/ TCCACATACAGTCCTGGATGATGATG	
Episomal Plasmids (RT-PCR)	*KLF4*	TTCCTGCATGGCAGAGGAGCCC/ AATGTATCGAAGGTGCTCAA	
Episomal Plasmids (RT-PCR)	*SeV*	GGATCACTAGGTGATATCGAGC/ ACCAGACAAGAGTTTAAGAGATATGTATC	
House-Keeping Genes (RT-PCR)	*GAPDH*	TGAAGGTCGGAGTCAACGGA/ CCAATTGATGACAAGCTTCCCG	