



## Generation of induced pluripotent stem cell lines from three individuals with autism spectrum disorder

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### ABSTRACT

Uncovering the molecular mechanisms of autism spectrum disorder (autism) necessitates development of relevant experimental models that are capable of recapitulating features of the clinical phenotype. Using non-integrative episomal vectors, peripheral blood mononuclear cells derived from three unrelated individuals diagnosed with autism were reprogrammed to induced pluripotent stem cells (iPSCs). The resultant lines exhibited the expected cellular morphology, karyotype, and evidence of pluripotency. These iPSCs constitute a valuable resource to support investigations of the underlying aetiology of autism.

### 1. Resource Table

(continued)		
Unique stem cell lines identifier	Type of cell lines Origin Additional origin info required	iPSCs Human <b>MONUi001</b> Age: 35 years, 4 months (at blood sampling) Sex: Male Ethnicity: Caucasian
Alternative name(s) of stem cell lines		<b>MONUi002</b> Age: 10 years, 7 months (at blood sampling) Sex: Female Ethnicity: Caucasian
Institution	Cell Source	<b>MONUi003</b> Age: 13 years, 4 months (at blood sampling) Sex: Male Ethnicity: Caucasian
Contact information of distributor	Clonality Method of reprogramming	Peripheral Blood Mononuclear Cells (PBMCs) Clonal, manually picked for expansion Episomal vector-based reprogramming (OCT4, SOX2, KLF4, MYC) NO N/A
	Genetic Modification Type of Genetic Modification	(continued on next page)

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(continued)

Evidence of the reprogramming transgene loss (including genomic copy if applicable)	Reverse transcription polymerase chain reaction (RT-PCR)
Associated disease	Autism spectrum disorder (autism)
Gene/locus	N/A
Date archived/stock date	10/6/2023
Cell line repository/bank	<a href="https://hpscreg.eu/cell-line/MONUi001-A">https://hpscreg.eu/cell-line/MONUi001-A</a> <a href="https://hpscreg.eu/cell-line/MONUi001-B">https://hpscreg.eu/cell-line/MONUi001-B</a> <a href="https://hpscreg.eu/cell-line/MONUi001-C">https://hpscreg.eu/cell-line/MONUi001-C</a> <a href="https://hpscreg.eu/cell-line/MONUi002-A">https://hpscreg.eu/cell-line/MONUi002-A</a> <a href="https://hpscreg.eu/cell-line/MONUi002-B">https://hpscreg.eu/cell-line/MONUi002-B</a> <a href="https://hpscreg.eu/cell-line/MONUi002-C">https://hpscreg.eu/cell-line/MONUi002-C</a> <a href="https://hpscreg.eu/cell-line/MONUi003-A">https://hpscreg.eu/cell-line/MONUi003-A</a> <a href="https://hpscreg.eu/cell-line/MONUi003-B">https://hpscreg.eu/cell-line/MONUi003-B</a> <a href="https://hpscreg.eu/cell-line/MONUi003-C">https://hpscreg.eu/cell-line/MONUi003-C</a>
Ethical approval	Monash University Human Research Ethics Committee (MUHREC), Project ID 25688

## 2. Resource utility

Autism is a highly heritable neurodevelopmental condition. However, its complete aetiology remains elusive due to inaccessibility of relevant living tissue and paucity of appropriate *in vivo/in vitro* models. Donor-derived iPSCs afford important tools for examining the molecular mechanisms driving disorder pathophysiology, permitting interrogation of the determinants of the clinical phenotype.

## 3. Resource details

Blood samples were collected via standard phlebotomy procedures from three unrelated donors with a primary diagnosis consistent with autism (Diagnostic and Statistical Manual, 5th edition; [American Psychiatric Association, 2013](#)). To assess eligibility, individuals completed a comprehensive battery of (1) self- and/or parent-reported psychiatric and behavioural questionnaires, (2) age-appropriate cognitive assessment, and (3) a structured/semi-structured diagnostic interview with a research practitioner (see [Supplementary Table S1](#) for further detail). Donor DNA were genetically screened to confirm an idiopathic origin of presenting symptomatology via molecular karyotype analysis (Illumina Infinium GSA-24, 0.5 Mb resolution) and Fragile X specific polymerase chain reaction (PCR) at the Victorian Clinical Genetics Services (VCGS; The Royal Children's Hospital, Melbourne, Australia). Where psychiatric, behavioural, cognitive, and/or genetic testing were conducted previously, historical reports were accessed. Data were collated and reviewed by a multidisciplinary team consisting of a qualified paediatrician, speech pathologist, and psychologist to confirm participant diagnosis.

Donor PBMCs isolated from whole blood were reprogrammed to iPSCs using episomal vectors expressing OCT4, SOX2, KLF4, and MYC transgenes. The resultant cells exhibited expected clonal morphology, as ascertained via bright field microscopy ([Fig. 1A](#)). Pluripotency was established qualitatively through immunocytochemical analysis of OCT4 and SSEA4 markers ([Fig. 1B](#)), and corroborated by expression of OCT3/4, TRA-1-60 and SSEA4 as detected by flow cytometry ([Fig. 1C](#)). The differentiation potential of the lines was demonstrated by successful formation of embryoid bodies (EBs) *in vitro*, with positive staining of endodermal (SRY-box 17, SOX17; Forkhead Box A2, FOXA2), mesodermal (Smooth Muscle Actin, SMA; Brachyury/Bry), and ectodermal

(Neuronal Nuclei, NeuN; Microtubule-associated Protein 2, MAPT2) markers ([Fig. 1D](#)). Loss of episomal reprogramming factors was confirmed by reverse transcription PCR ([Supplementary Fig. 1](#)), indicating that the iPSCs were vector- and transgene-free at passage 5. Commercial testing via PCR (Cerberus Sciences, Adelaide, Australia) demonstrated that the lines were free from mycoplasma contamination (reports with authors). Molecular karyotyping of the reprogrammed lines by the VCGS revealed the expected karyotypes without aneuploidy (MONUi001: arr(X,Y)x1,(1-22)x2; MONUi002: arr(X,1-22)x2; MONUi003: arr(X,Y)x1,(1-22)x2) (available with journal). Comparative analyses between array data from the iPSCs and their respective parental PBMC lines demonstrated 99.99% concordance, authenticating identity (available with journal). The analyses performed for the characterisation and validation of all iPSCs generated are summarised in [Table 1](#).

## 4. Materials and methods

### 4.1. PBMC reprogramming and iPSC culturing

Blood samples were collected in K2-EDTA treated tubes (BD Vacutainer) and PBMCs isolated by Ficoll-Paque Plus (Cytiva) gradient centrifugation.  $2\text{--}4 \times 10^6$  cells were cryopreserved in 10% DMSO and 90% FBS until reprogramming.

$1 \times 10^6$  recovered PBMCs were plated in StemSpan Serum-Free Expansion Medium II (SFEM) supplemented with cytokines (StemSpan Erythroid Expansion Supplement, StemCell Technologies) for expansion. Media changes were performed daily by centrifugation at  $300 \times g$  for 5 min at room temperature (RT), resuspension of the cell pellet in 2 mL supplemented SFEM, and replating.

After 7–9 days, cells were centrifuged at  $400 \times g$  at RT for 5 min, counted, and resuspended in Resuspension Buffer T (Neon Transfection System, Invitrogen) to  $1 \times 10^7$  cells/mL. Cells were transfected with an episomal vector mix containing 0.3 µg of MOS (expressing OCT3/4 and SOX2; Addgene #64120), MXB (expressing BCL2L1; Addgene #64122), pEV-SFFV-KLF4 (expressing KLF4; [Wen et al., 2018](#)), and pEV-SFFV-MYC (expressing MYC; [Wen et al., 2018](#)) plasmids and 0.5 µg EBNA1 mRNA using the Neon Transfection System (1050 V, 30 ms X2; Invitrogen). Cells were immediately plated in supplemented SFEM on culture vessels pre-coated with hESC-qualified Matrigel (1:200, Corning) and irradiated Mouse Embryonic Fibroblasts, then transitioned to Essential 8 media (E8, Invitrogen) through gradual media substitution. Colonies were manually picked and transitioned to Matrigel-coated vessels 7–8 days post-transfection. Passaging was performed every 2–4 days at a 1:2 ratio using 0.5 mM EDTA + DPBS without a survival promoter.

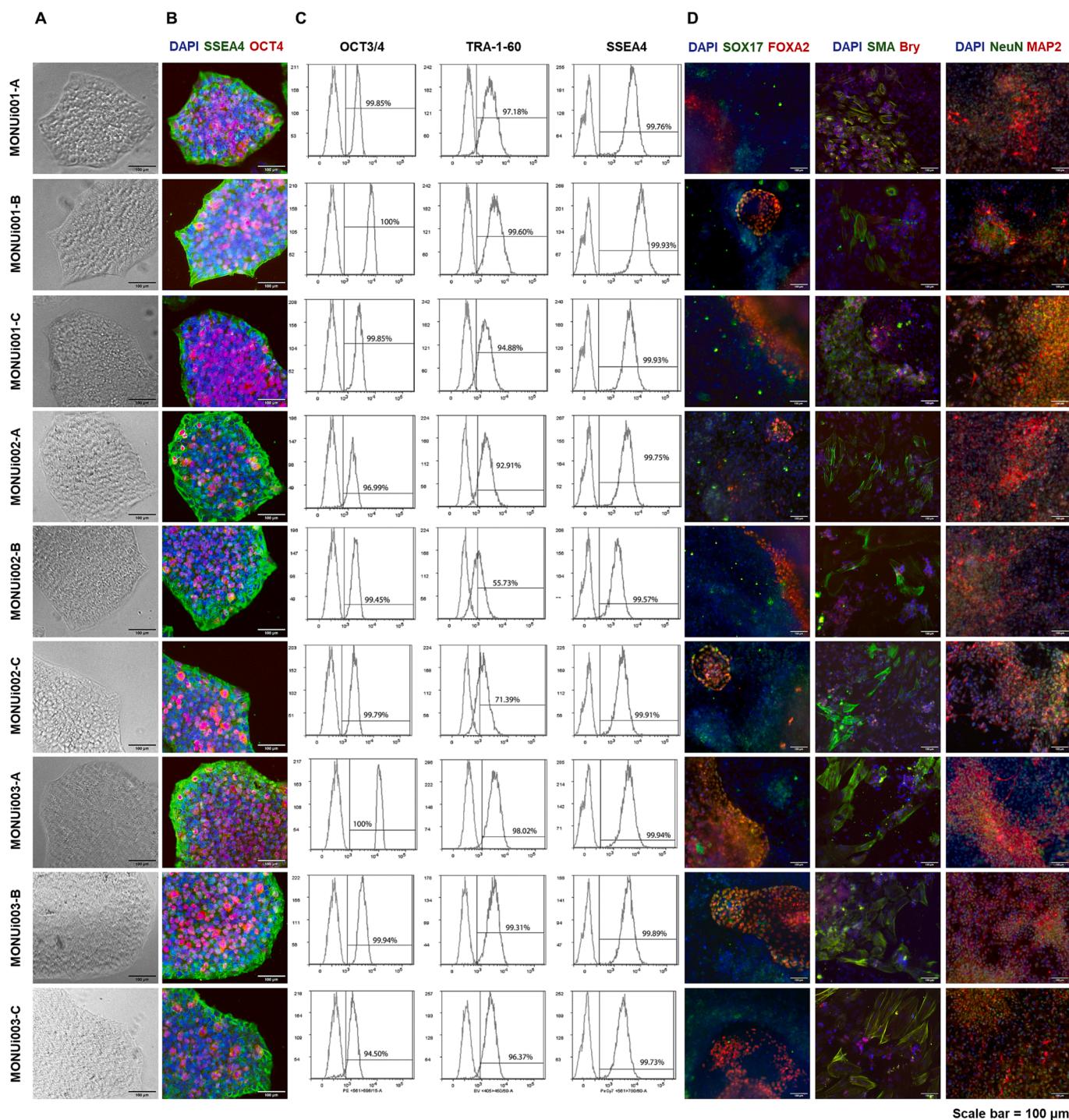
Cells were cultured at 37°C, 5% CO<sub>2</sub> in a humidified incubator throughout.

### 4.2. Detection of episomal vectors

Loss of reprogramming vectors was confirmed via reverse transcription PCR using primers listed in [Table 2](#). Total RNA was isolated at passage 5+ and cDNA synthesised using the Tetro cDNA Synthesis Kit (Bioline). gDNA derived from vector-free iPSCs and 1 pg of reprogramming plasmid vectors were provided as negative and positive controls, respectively.

### 4.3. Flowcytometry and immunostaining of pluripotency markers

Cells (passage 10–12) were harvested using Tryples (Gibco) then incubated with conjugated surface antibodies for 10 min on ice or conjugated intracellular antibodies for 30 min at 4 °C. Intracellularly stained cells were fixed and permeabilised using the eBioscience Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific). Propidium iodide (Sigma) was added to surface-stained cells, and flow cytometric analysis performed using a LSRFortessa x20 flow cytometer



Scale bar = 100 μm

Fig. 1.

(BD Bioscience).

Pluripotency marker staining was performed at passage 9 + using the Pluripotent Stem Cells 4-Marker Immunocytochemistry Kit (Invitrogen). Imaging was conducted using the EVOS M5000 Imaging System (ThermoFisher Scientific).

#### 4.4. SNP array

PBMC and iPSC (passage 10–12) karyotyping was conducted using the Illumina Infinium GSA-24 Array v3.0. Data were compared to human reference sequence GRCh38/hg38 (Dec 2013).

SNPduo comparative analysis (Roberson & Pevsner, 2009) of array data was performed to authenticate iPSC identity.

#### 4.5. EB formation and immunofluorescence

EBs were formed and plated *in vitro* as previously described (Bozoglu et al., 2019). EBs were fixed with 4% Formaldehyde for 10 min then permeabilised in 0.2% Triton X-100 (Sigma-Aldrich) for 10 min before blocking in 2% bovine serum albumin (Sigma-Aldrich) for 1 h at RT. EBs were incubated with primary antibodies overnight at 4 °C then with secondary antibodies for 60 min at RT (Table 2) before

**Table 1**  
Characterization and validation.

Classification	Test	Result	Data
<b>Morphology Phenotype</b>	Bright field microscopy	Normal	<a href="#">Fig. 1</a> , Panel A ( <a href="#">Fig. 1A</a> )
	Qualitative analysis	Positive immunostaining for pluripotency markers OCT4 and SSEA4	<a href="#">Fig. 1</a> , Panel B ( <a href="#">Fig. 1B</a> )
	Quantitative analysis	Expression of TRA-1-60, SSEA4, and OCT3/4 was validated by flow cytometry	<a href="#">Fig. 1</a> , Panel C ( <a href="#">Fig. 1C</a> )
<b>Genotype</b>	Illumina Infinium GSA-24 Array	MONUi001: arr(X,Y)x1,(1-22)x2 MONUi002: arr(X,1-22)x2 MONUi003: arr(X,Y)x1,(1-22)x2 Resolution 0.50 Mb	Submitted in archive with journal
<b>Identity</b>	Illumina Infinium GSA-24 Array OR	>99.9% concordance at 650,000 SNP markers determined via SNPduo comparative analysis	Submitted in archive with journal
<b>Mutation analysis (IF APPLICABLE)</b>	STR analysis	N/A	N/A
	Sequencing	N/A	N/A
	Southern Blot OR WGS	N/A	N/A
<b>Microbiology and virology</b>	Mycoplasma	Negative mycoplasma testing via PCR	Available with the authors
<b>Differentiation potential</b>	Embryoid body formation	Expression of appropriate markers for each of the three germ layers (i.e., ectoderm, endoderm, mesoderm)	<a href="#">Fig. 1</a> , Panel D ( <a href="#">Fig. 1D</a> )
<b>List of recommended germ layer markers</b>	Expression of markers at the protein level (immunofluorescence)	Ectoderm: NeuN, MAP2 Endoderm: SOX17, FOXA2 Mesoderm: SMA, Brachyury/Bry	Immunofluorescence
<b>Donor screening (OPTIONAL)</b>	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
<b>Genotype additional info (OPTIONAL)</b>	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

**Table 2**  
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry				
	Antibody	Dilution	Company Cat #	RRID
Pluripotency Markers	Rabbit anti-OCT4	1:100	ThermoFisher Scientific Cat#A24867	AB_2650999
Pluripotency Markers	Mouse anti-SSEA4 (IgG3)	1:100	ThermoFisher Scientific Cat#A24866	AB_2651001
Pluripotency Markers	BV421 anti-human TRA-1-60 antibody	1:100	BD Biosciences Cat#562711	AB_2737738
Pluripotency Markers	PE/Cy7 anti-human SSEA-4 antibody	1:100	BioLegend Cat#330420	AB_2629631
Pluripotency Markers	PE anti-human Oct3/4 antibody	1:100	ThermoFisher Scientific Cat#12584180	AB_914364
Differentiation Markers	Rabbit anti-NeuN	1:500	Abcam Cat#ab177487	AB_2532109
Differentiation Markers	Chicken anti-MAP2	1:2500	Abcam Cat#ab92434	AB_2138147
Differentiation Markers	Goat anti-SOX17	1:100	R and D Systems Cat#AF1924	AB_355060
Differentiation Markers	Rabbit anti-FOXA2	1:100	Abcam Cat#ab256493	AB_2924654
Differentiation Markers	Mouse anti-SMA	1:25	Agilent Cat#M0851	AB_2223500
Differentiation Markers	Rabbit anti- Brachyury/Bry	1:1000	Abcam Cat#ab209665	AB_2750925
Secondary Antibodies	Donkey Anti-Rabbit, Alexa Fluor 555	1:250	ThermoFisher Scientific Cat#A24869	AB_2651006
Secondary Antibodies	Goat anti-mouse IgG3, Alexa Fluor 488	1:250	ThermoFisher Scientific Cat#A24877	AB_2651008
Secondary Antibodies	Goat anti-rabbit IgG H&L, Alexa Fluor 488	1:500	Abcam Cat#ab150081	AB_2734747
Secondary Antibodies	Goat anti-rabbit IgG H&L, Alexa Fluor 555	1:500	Abcam Cat#ab150078	AB_2722519
Secondary Antibodies	Goat anti-chicken IgY H&L, Alexa Fluor 555	1:500	Abcam Cat#ab150174	AB_2864276
Secondary Antibodies	Donkey anti-goat IgG H&L, Alexa Fluor 488	1:500	Abcam Cat#ab150133	AB_2832252
Secondary Antibodies	Goat anti-mouse IgG H&L, Alexa Fluor 488	1:500	Abcam Cat#ab150117	AB_2688012
<b>Primers</b>				
Target				
Episomal Plasmids (RT-PCR)	<i>ampR</i> gene	350 bp	Forward/Reverse primer (5'-3')	
Episomal Plasmids (RT-PCR)	oriP region	250 bp	CAGTCCTATTAAATTTGCCGG/GCTATGTGGCGCCGTATTAT AGCTACCGATAAGCGGACC/CCCTCGTGAATCTGACC	

counter staining with NucBlue/DAPI (Invitrogen). Images were captured using the EVOS M5000 Imaging System.

#### 4.6. Mycoplasma testing

Mycoplasma testing was conducted commercially by Cerberus Sciences (Adelaide, Australia).

#### 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.

#### Acknowledgments

The authors would like to thank the donors and their families for their generous donation of time and participation.

The authors acknowledge the facilities, and the scientific and technical assistance of Murdoch Children's Research Institute iPSC and GE Core Facility. MCRI iPSC and GE Core Facility was established using a generous donation from the Stafford Fox Medical Research Foundation. It is currently supported by Phenomics Australia (PA), and the Novo Nordisk Foundation reNEW Center for Stem Cell Medicine (NNF21CC0073729). PA is supported by the Australian Government through the National Collaborative Research Infrastructure Strategy (NCRIS) program.

## Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. KP is supported by an Australian Government Research Training Program (RTP) Scholarship. MAB is supported by a Senior Research Fellowship from the National Health and Medical Research Council (NHMRC) of Australia.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2023.103170>.

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