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Lab resource: Stem Cell Line

Generation of a miR-26b stem-loop knockout human iPSC line, MCRIi019-A-1, using CRISPR/Cas9 editing

Louise H.W. Kung [a], Lisa Sampurno [a], Christopher B. Little [b], Shireen R. Lamandé [a,c], John F. Bateman [a,c,*]

[a] Murdoch Children's Research Institute, Parkville, Victoria, Australia
[b] Raymond Purves Bone and Joint Research Laboratories, Kolling Institute of Medical Research, Institute of Bone and Joint Research, University of Sydney, St Leonards, New South Wales, Australia
[c] Department of Paediatrics, University of Melbourne, Australia

A B S T R A C T

miR-26b has been implicated in a wide range of human diseases, including cancer, diabetes, heart disease, Alzheimer's disease and osteoarthritis. To provide a tool to explore the importance of miR-26b in this broad context, we have generated and characterized a homozygous miR-26b stem-loop knockout human iPSC line. This gene-edited line exhibited a normal karyotype, expressed pluripotency markers and differentiated into cells representative of the three embryonic germ layers. This iPSC line will be valuable for studies investigating disease mechanisms and testing therapeutic strategies *in vitro*.

Resource Table

Unique stem cell line identifier	MCRIi019-A-1
Alternative name(s) of stem cell line	1502.3 miR-26b KO
Institution	Murdoch Children's Research Institute, Melbourne, Australia
Contact information of distributor	Professor John Bateman john.bateman@mcri.edu.au
Type of cell line	iPSC
Origin	Human
Additional origin info	ATCC cell line: CRL-1502 Age: 12 weeks gestation Sex: Female Ethnicity if known: Black
Cell Source	Dermal fibroblast-derived human induced pluripotent cell line MCRIi019-A (http://hpscreg.eu/cell-line/MCRIi019-A)
Clonality	Clonal
Method of reprogramming	Episomal vectors
Genetic Modification	Yes
Type of Modification	Knockout/deletion
Associated disease	N/A
Gene/locus	MIR26B/2q.35
Method of modification	CRISPR/Cas9
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	October 2018
Cell line repository/bank	http://hpscreg.eu/cell-line/MCRIi019-A-1
Ethical approval	This study was approved through the Human Research Ethics Committee of the Royal Children's Hospital, Victoria, Australia (HREC #33118)

1. Resource utility

This homozygous miR-26b knockout iPSC line, together with its parental isogenic control line, will provide an *in vitro* human model system to explore the role of miR-26b in development and in a broad range of diseases. This will provide an experimental platform to address fundamental developmental questions and illuminate the pathological pathways downstream of miR-26b dysregulation in iPSC-derived human organoid disease models.

2. Resource details

microRNAs (miRs) are a large family of small non-coding RNAs which modulate gene expression at the post-transcriptional level in a wide range of developmental and physiological contexts. In addition to the role of miRs in stem cell reprograming, pluripotency and differentiation, miR dysregulation is increasingly relevant in disease

* Corresponding author at: Murdoch Children's Research Institute, Parkville, Victoria, Australia.
E-mail address: john.bateman@mcri.edu.au (J.F. Bateman).

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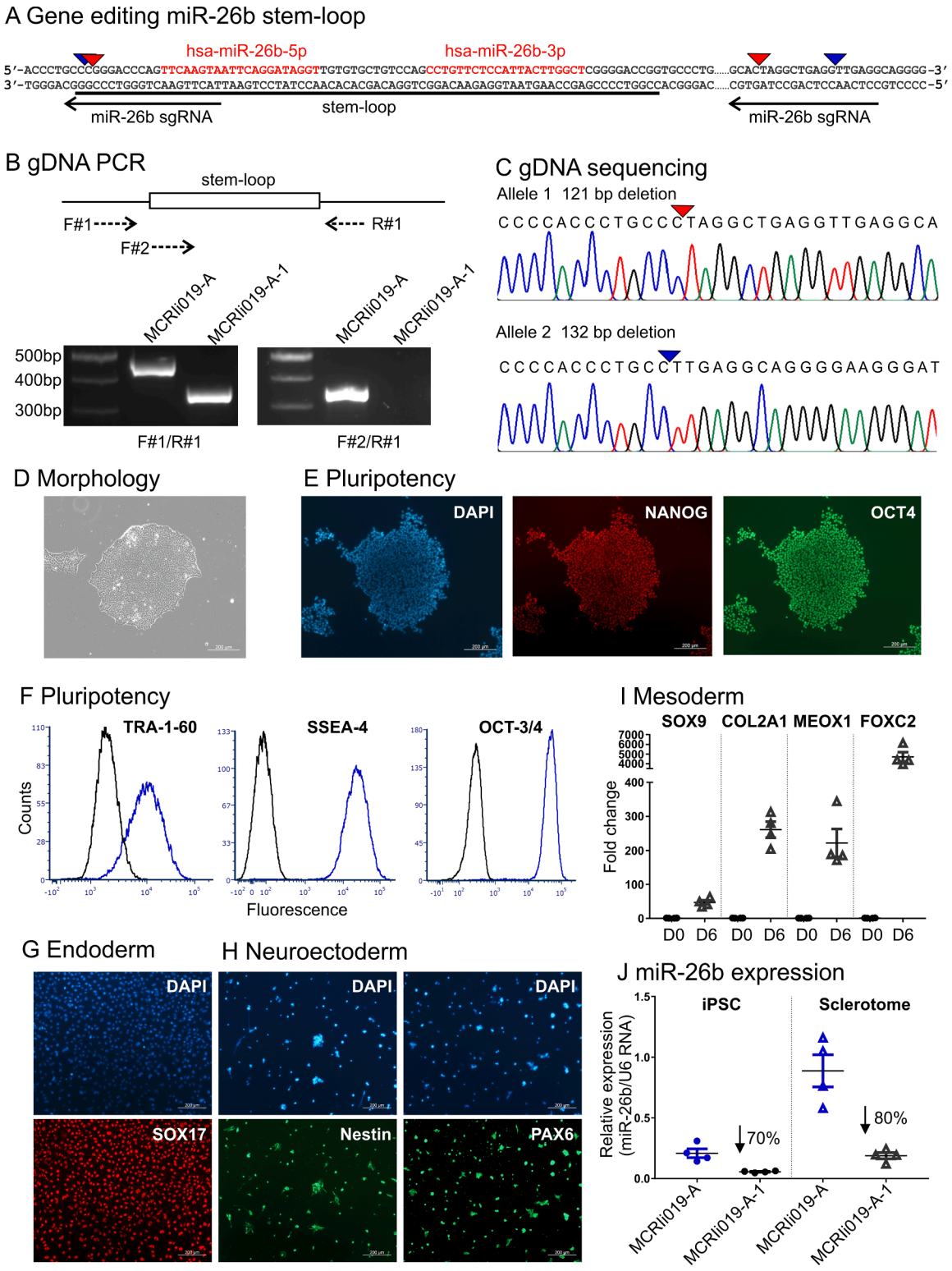

Fig. 1. Cellular and molecular characterization. A. Target gene sequence for CRISPR/Cas9 editing to delete the miR-26b stem-loop region (underlined). sgRNA locations are shown and the position of the breakpoints for each allele are indicated by red and blue arrows. B. Diagram showing the positions of the primer pairs used to screen for the stem-loop deletion. PCR showing extent of the deletion with forward and reverse primer #1, and confirming the absence of the stem loop in the mutant using forward primer #2 and reverse primer #1. Primer details are provided in Table 2. C. Sanger sequencing demonstrating the breakpoints of the deletion in both alleles. D, MCRIi019-A-1 morphology by bright field microscopy (scale bar 200 μm). E. Immunohistochemistry showing expression of pluripotency markers NANOG and OCT4 (scale bar 200 μm). F. FACS analysis of iPSC for pluripotency markers TRA-1–60, SSEA-4 and OCT3/4. G, H. Immunohistochemistry showing the ability of MCRIi019-A-1 to differentiate into endoderm (SOX17) and neuroectodermal (Nestin, PAX6) lineages (scale bar 200 μm). I. Mesodermal differentiation shown by marker gene mRNA expression (*SOX9, COL2A1, MEOX1* and *FOXC2*) in the transition of iPSC (D0) to sclerotome (D6). qPCR was used to measure mRNA expression relative to *ACTB*, presented as fold change from D0 iPSC. J. qPCR showing miR-26b-5p expression (relative to U6 RNA expression) in the gene edited MCRIi019-A-1 line compared to the parental line MCRI019-A. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1, panel D
Phenotype	Qualitative analysis	OCT4 and NANOG positive staining	Fig. 1, panel E
	Quantitative analysis	MCRIi019-A-1: TRA-1–60: 81.8% SSEA4: 99.0% OCT3/4: 98.1%	Fig. 1, panel F
Genotype	Molecular Karyotype and resolution	arr(1–22,X)x2; 46XX Resolution: 0.5 Mb	Supplementary Fig. 1
Identity	SNPDuo analysis of SNParrays	Identical genotypes (>99.9%) for the entire genome, indicating the lines are from the same individual	Supplementary Fig. 1
Mutation analysis	Sequencing	Homozygous deletion of miR-26b stem-loop	Fig. 1, panel B&C
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR. Negative	Supplementary Fig. 2
Differentiation potential	Directed differentiation	Endoderm: SOX17	Fig. 1, panel G
		Ectoderm: Nestin and PAX6	Fig. 1, panel H
		Mesoderm: MEOX1, FOXC2, SOX9, COL2A1	Fig. 1, panel I
Donor screening	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

pathogenesis. One of these, miR-26b, is dysregulated in a range of human diseases, including cancer, diabetes, heart disease, Alzheimer's disease and osteoarthritis. To produce a human *in vitro* cell model system to explore the role of miR-26b in various developmental and disease contexts we have generated a miR-26b knockout human iPSC line.

Control iPSCs derived from dermal fibroblasts (ATCC cat: CRL-1502; http://hpscreg.eu/cell-line/MCRIi019-A) by the introduction of integration-free episomal vectors (Kung et al., 2020) were transfected with plasmids encoding miR-26b-specific sgRNAs (Fig. 1A) and Cas9 mRNA (Howden et al., 2018). To identify potential knockout clones, individual iPSC colonies were screened by PCR using primers flanking the miR-26b stem-loop sequence, demonstrating the ~120–130 bp deletion in MCRi019-A-1 compared to the unedited parental line, MCRIi019-A (Fig. 1B). Further PCR screening was performed using primers within the stem-loop to confirm the deletion and clonality of MCRi019-A-1 (Fig. 1B). To assess the precision of the deletion introduced by gene editing, PCR amplicons were generated using primers that bind either side of the miR-26b stem-loop target site (Fig. 1B). Sanger sequencing of the PCR products confirmed the break points and excision of 121 bp from allele 1 and 132 bp from allele 2, removing the sequences containing miR-26b-5p and miR-26b-3p in both alleles (Fig. 1C).

MCRIi019-A-1 colonies displayed normal stem cell morphology with tightly packed cells, well-defined boundaries and a high nucleus to cytoplasm ratio (Fig. 1D). Immunofluorescent staining confirmed the co-expression of nuclear pluripotency markers, OCT4 and NANOG (Fig. 1E) and flow cytometry showed that MCRIi019-A-1 cells strongly express the pluripotency markers OCT-3/4, SSEA4 and TRA-1-60 (Fig. 1F). The ability of MCRIi019-A-1 to differentiate into the three main germ layers was confirmed. Directed differentiation to definitive endoderm (Loh et al., 2014) induced expression of the endoderm marker SOX17 (Fig. 1G). Neuroectoderm differentiation was demonstrated by co-expression of Nestin and PAX6 (Fig. 1H). Lastly, *SOX9, COL2A1,*

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Marker	Oct-4A (C30A3) Rabbit Monoclonal Antibody	1:400	Cell Signaling Technology Cat# 2840S, RRID: AB_2167691
Pluripotency Marker	Purified anti-Nanog Antibody	1:200	BioLegend Cat# 674202, RRID: AB_2564574
Endoderm Marker	Human Sox17 Antibody	1:100	R&D Systems Cat# AF1924, RRID: AB_355060
Neuroectoderm Marker	PAX6 Monoclonal Antibody (13B10-1A10)	1:200	ThermoFisher Scientific Cat#MA1-109; RRID: AB_2536820
Neuroectoderm Marker	Anti-Nestin Antibody, clone 10C2	1:200	Merck Cat# MAB5326, RRID: AB_2251134
Secondary Antibody	Donkey anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	1:1000	ThermoFisher Scientific Cat# A21203, RRID: AB_2535789
Secondary Antibody	Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:1000	ThermoFisher Scientific Cat# A11008, RRID: AB_ 143,165
Secondary Antibody	Donkey Anti-Goat IgG H&L (Alexa Fluor® 594)	1:1000	Abcam Cat# ab150132, RRID: AB_2810222
Secondary Antibody	Goat anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:1000	ThermoFisher Scientific Cat# A11029; RRID: AB_2534088
FACS Antibody	BV421 Mouse Anti-Human TRA-1–60 Antigen	1:20	Becton Dickinson Cat# 562711, RRID: AB_2737738
FACS Antibody	Alexa Fluor 647 anti-human SSEA-4 antibody	1:100	BioLegend Cat# 330408, RRID: AB_1089200
FACS Antibody	PE Mouse anti-Oct3/4	1:5	BD Biosciences Cat# 560186, RRID: AB_1645331
Primers			
	Target	**Forward/Reverse primer (5′-3′)**	
mir-26b sgRNA	mir-26b stemloop	ACTTGAACTGGGTCCCGGGC/ CCTCAACCTCAGCCTAGTGC	
F#1/R#1	mir-26b stemloop	GCTTGTGCTGACTCCAAGCC/ GACGCTTCAACACGTAGACC	
F#2	mir-26b stemloop	GTTCTCCATTACTTGGCTCGG	

(*continued on next page*)

Table 2 (*continued*)

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
mir-26b EcoRI	mir-26b stemloop		CAGTGAATTCTGTCCCAAAGTCACACAGAACC/ CAGTGAATTCAGGACGCTTCAACACGTAGACC
pSMseq	pSMART-EcoRI vector		CAGTCCAGTTACGCTGGAGTC/ GGTCAGGTATGATTTAAATGGTCAGT
Cartilage marker	SOX9		AAGTCGGTGAAGAACGGGC/ TCTCGCTTCAGGTCAGCCTT
Cartilage marker	COL2A1		TCACGTACACTGCCCTGAAG/ GCCCTATGTCCACACCGAAT
Sclerotome marker	MEOX1		ACTCGGCTCCGCAGATATGA/ GAACTTGGAGAGGCTGTGGA
Sclerotome marker	FOXC2		TGGTATCTCAACCACAGCGG/ CCCGGGACACGTCAGTATTT
Housekeeping gene	ACTB		AAGTCCCTTGCCATCCTAAAA/ ATGCTATCACCTCCCCTGTG

MEOX and *FOXC2* mRNA expression (Fig. 1I) confirmed MCRIi019-A-1 differentiation into sclerotome, a mesoderm derivative, by day 6 (D6) of the differentiation protocol (Loh et al., 2016). qPCR of miR-26b-5p expression in control MCRIi019-A and MCRIi019-A-1 confirmed miR-26b knockout at the RNA level in iPSC and differentiated sclerotome cells (Fig. 1J). Genome SNP array analysis of MCRIi019-A-1 confirmed no aneuploidies or large deletions or insertions (Table 1, Supplementary Fig. 1). It should be noted that the SNP analysis does not preclude the presence of balanced translocations. SNPDuo analysis confirmed that MCRIi019-A-1 had > 99% identity with the parental line, MCRIi019-A (Table 1, Supplementary Fig. 1). MCRIi019-A-1 was free of mycoplasma contamination (Table 1, Supplementary Fig 2).

3. Materials and methods

3.1. Cell culture

The human iPSC lines were cultured at 37 °C with 5% CO_2 on Matrigel (Corning) coated plates in Essential 8 (E8) medium (Thermo Fisher Scientific). Media was changed daily and cells were passaged (1:4–1:6 split) every 3–4 days with 0.5 mM EDTA in PBS (Thermo Fisher).

3.2. CRISPR/Cas9-mediated gene editing

The CRISPR design tool (http://crispr.mit.edu/) was used to design sgRNAs flanking the miR-26b stem-loop sequence. The sgRNA oligonucleotides (Fig. 1A, Table 2) were annealed and ligated into pSMART-sgRNA (Sp) plasmids (Addgene # 80427). Control MCRIi019-A iPSCs were harvested with TrypLE (Thermo Fisher Scientific) 2 days after passaging and 1×10^6 cells were transfected with 5 μg of *in vitro* transcribed Cas9 mRNA (Howden et al., 2018) and 2 μg of the pSMART-miR26b-sgRNA plasmids using the Neon® Transfection System (Thermo Fisher Scientific). Cells were electroporated (1100 V, 30 ms, 1 pulse) as previously described (Howden et al 2018) and plated over 4 wells of a Matrigel-coated 6-well plate in E8 medium with 10 μM ROCK inhibitor, Y-27632 (Tocris). The medium was switched to E8 without Y-27632 the next day and changed every other day. Individual colonies were isolated and expanded in E8 medium.

3.3. PCR screening and sequencing

gDNA was extracted from potentially targeted clones using a DNAeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. All PCR was performed using GoTaq Green Mastermix (Promega) with using an Applied Biosystems (Veriti) 96-well thermocycler. PCR conditions were 95 °C for 3 min, followed by 35 cycles of 95 °C for 18 s, 55 °C for 18 s, 72 °C for 40 s then 72 °C for 5 min. PCR products were analysed by agarose gel electrophoresis. Primers flanking the miR26b stem-loop (Fig. 1B, Table 2) were used to screen individual colonies and the deletion was further confirmed using primers within the stem-loop (Fig. 1B, Table 2). To assess the break points in the miR-26b knockout clones, PCR amplicons were generated using primers containing EcoRI cut sites (Table 2; miR-26b EcoRI) that bind either side of the miR-26b stem-loop target site. PCR products were cloned into linearised pSMART-EcoRI vectors and both alleles were Sanger sequenced to identify clones containing the miR-26b deletion.

3.4. Quantitative RT-PCR

RNA was extracted using TRIzol (Invitrogen) and reverse transcribed using QuantiTect® Reverse Transcription Kit (Qiagen). qPCR was performed using Brilliant III Ultra-Fast SYBR®Green qPCR Master Mix (Agilent Technologies) and gene-specific primers (*SOX9, COL2A1, MEOX1, FOXC2*; Table 2) and expression was measured relative to the house-keeping gene *ACTB*. For miR-26b expression, samples were reverse transcribed using miScript II RT kit (Qiagen) and qPCR was performed using miScript SYBR Green PCR and primer assays (Qiagen). miR-26b-5p expression was quantified relative to U6 RNA expression.

3.5. Flow cytometry

Cells were dissociated with TrypLE (Thermo Fisher Scientific) and incubated with conjugated antibodies to cell surface proteins TRA-1–60 and SSEA4 (Table 2) diluted in PBS containing 2% fetal bovine serum (FBS) for 15 min at 4 °C. Cells were washed with 2% FBS in PBS, then fixed and permeabilized using the eBioscience™ Foxp3/Transcription Factor Staining buffer set (Thermo Fischer Scientific), then stained with a conjugated antibody to intracellular OCT3/4 (Table 2). Samples were analysed using a LSR Fortessa X20 (BD Biosciences) and BD FACSDiva and FCS Express software.

3.6. Directed differentiation

iPSCs were differentiated in monolayer culture into definitive endoderm for 5 days (Loh et al., 2014), anterior neuroectoderm for 12 days (Tchieu et al., 2017) and sclerotome for mesodermal potential for 6 days (Loh et al., 2016). Differentiation was assessed by immunocytochemistry and/or qPCR for lineage-specific markers.

3.7. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde, followed by permeabilization with 0.05% Triton X-100 in PBS for 10 min at 4 °C. Blocking was carried out in 3% bovine serum albumin in 0.1% PBST for 30 min at room temperature. Cells were incubated with primary antibodies at 4 °C overnight, followed by secondary antibodies for 60 mins at room temperature (Table 2). Cells were incubated with DAPI to counterstain nuclei prior to visualization by fluorescent microscopy (Olympus IX70).

3.8. Molecular karyotyping and SNP analysis

Cell pellets (MCRIi019-A-1, passage 21 and parental line MCRIi019-A, passage 10) were provided to the Victorian Clinical Genetics Service (Murdoch Children's Research Institute, Melbourne, Australia) and genomic DNA was analysed using an Infinium GSA-24 v1.0 SNP array (Illumina).

3.9. Mycoplasma detection

Absence of mycoplasma contamination in MCRIi019-A-1 was confirmed by PCR by the commercial service provider 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.

Acknowledgements

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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.2020.102118.

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