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Lab resource: Stem Cell Line

Generation of a heterozygous *COL1A1* (c.3969_3970insT) osteogenesis imperfecta mutation human iPSC line, MCRIi001-A-1, using CRISPR/Cas9 editing

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A B S T R A C T

To develop a disease model for the human 'brittle bone' disease, osteogenesis imperfecta, we have used gene editing to produce a facsimile of the patient heterozygous *COL1A1* mutation in an established control iPSC line. The gene-edited line had a normal karyotype, expressed pluripotency markers and differentiated into cells representative of the three embryonic germ layers. This iPSC line and the isogenic parental iPSC line will be of use in exploring osteogenesis imperfecta disease mechanisms and therapeutic approaches *in vitro*.

Resource table

Unique stem cell line identifier	MCRIi001-A-1
Alternative name(s) of stem cell line	MCRIi001-A-OI26; PB001-OI26
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	Age: 60 Sex: Male Ethnicity: Caucasian
Cell source	Peripheral blood mononuclear cells derived human induced pluripotent cell line MCRIi001-A (https://hpscreg.eu/cell-line/MCRIi001-A)
Clonality	Clonal
Method of reprogramming	Transgene free Sendai Virus
Genetic modification	Yes
Type of modification	Induced mutation
Associated disease	Osteogenesis imperfecta
Gene/locus	*COL1A1* c.3969dupT
Method of modification	CRISPR/Cas9
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	January 2017
Cell line repository/bank	https://hpscreg.eu/cell-line/MCRIi001-A-1
Ethical approval	RCH Human Research Ethics Committee 35121A

Resource utility

This heterozygous OI26 iPSC line will provide an *in vitro* human OI model to scrutinize the molecular pathology of disease and promote the discovery of new therapeutic strategies for OI.

Resource details

The 'brittle-bone' disorder, osteogenesis imperfecta (OI), is commonly caused by heterozygous mutations in the genes for type I collagen, *COL1A1* and *COL1A2*. To develop an OI iPSC disease modelling system for disease mechanism and therapeutic studies we introduced a patient OI mutation into the control iPSC line (MCRIi001-A; https://hpscreg.eu/cell-line/MCRIi001-A) to generate an isogenic OI iPSC line (MCRIi001-A-1). The patient mutation, OI26, was a heterozygous single base insertion in *COL1A1* exon 49 (c.3969_3970insT) which leads to a codon reading frameshift, introducing a premature stop codon (https://oi.gene.le.ac.uk/ DB-ID COL1A1_00359; PMID:2500431). This truncates and disrupts the critical collagen protein propeptide domain

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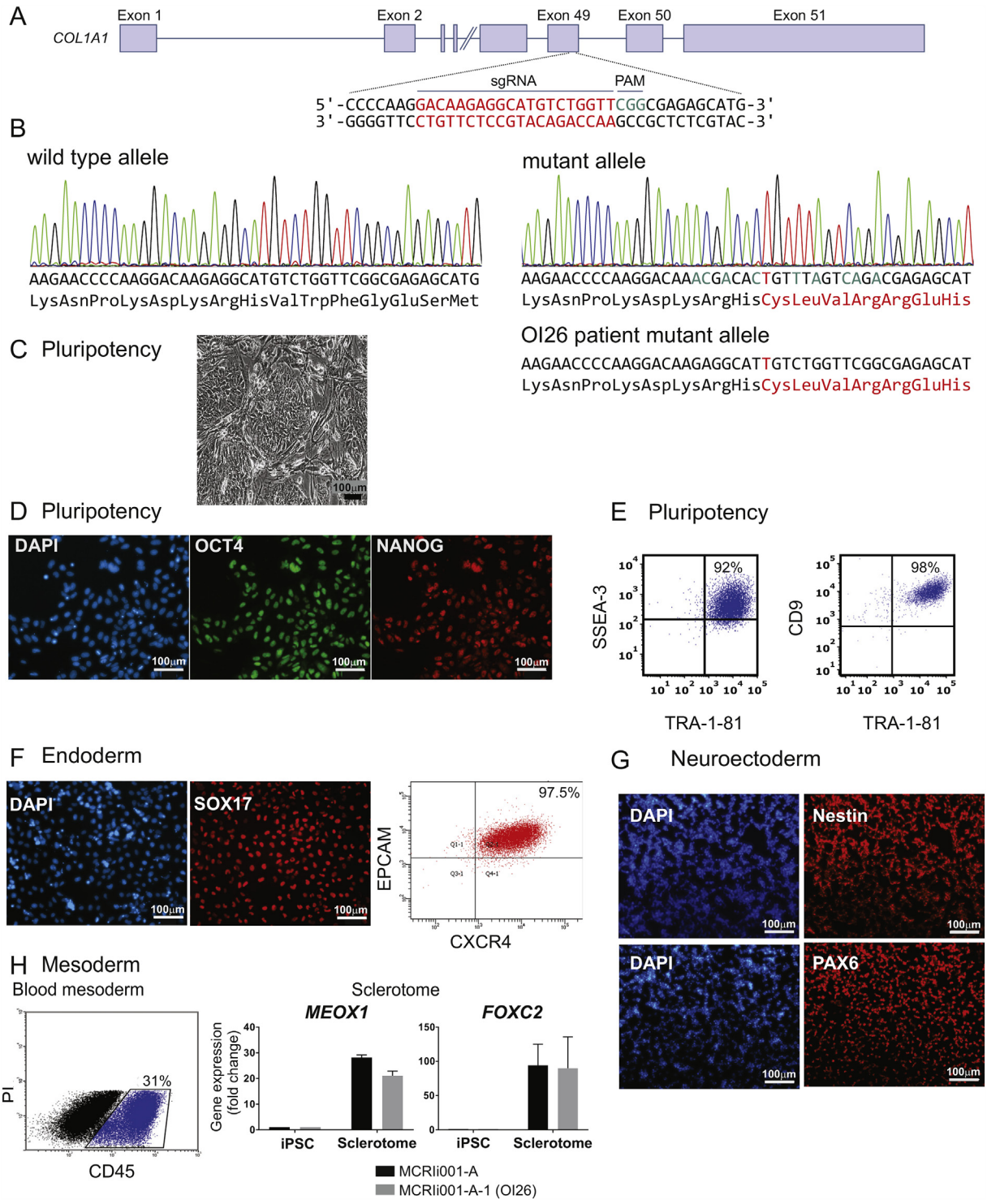

Fig. 1. Cellular and molecular characterization of MCRIi001-A-1.

which inhibits collagen trimerization.

Control MCRIi001-A iPSCs were transfected with sgRNA (Fig. 1A) and a double stranded mutation template containing synonymous base changes (Fig. 1B; shown in green in the mutant allele) to facilitate PCR screening. Cells were co-transfected with Cas9-Gem RNA, an approach that improves targeting efficiency and reduces unwanted mutations at the targeted locus (Howden et al., 2016) and with pEFBOS-GFP to allow single cell sorting. After sorting, heterozygous iPSC clones were identified by PCR screening using primers specific for wild-type and mutant alleles (Supplementary Fig. 1). Sequencing of clone MCRIi001-A-1 (Fig. 1B) confirmed the OI26 patient mutation had been introduced into one allele and there were no mutations in the other allele around the targeted site. Sequencing the mRNA expressed by MCRIi001-A-1 confirmed *COL1A1* mutant and wild-type allele expression (data not shown).

Genome SNP array analysis confirmed no aneuploidies or large deletions or insertions and > 99.9% identity of MCRIi001-A-1 with the parental line (Table 2, Supplementary Fig. 2). It should be noted that the SNP analysis does not preclude the presence of balanced translocations. MCRi001-A-1 was free from mycoplasma contamination

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel C
Phenotype	Qualitative analysis (Immunofluorescence)	OCT4 and NANOG Positive	Fig. 1 panel D
	Quantitative analysis (Flow cytometry)	SSEA-3 and TRA-1-81: 92% CD9 and TRA-1-81: 98%	Fig. 1 panel E
Genotype	SNP array (resolution 0.5 Mb)	46XY	Supplementary Data
Identity	Genetic analysis	SNPduo of SNParrays to compare parental and gene edited clones. Identical genotypes (> 99.9%) for the entire genome, indicating the lines are from the same individual	Available with the authors
Mutation analysis	Sequencing	Heterozygous mutation OI mutation confirmed. *COL1A1* (c.3969_3970insT)	Fig. 1 panel B
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR. Negative	Supplementary Data
Differentiation potential	Directed differentiation (Immunofluorescence, Flow cytometry and qRT-PCR)	Endoderm: SOX17, CXCR4 and EpCAM. Ectoderm: Nestin, and PAX6. Mesoderm (Blood mesoderm); CD45: (Sclerotome); MEOX1, FOXC2	Fig. 1 panels F,G,H

(Table 2, Supplementary Fig. 3).

MCRI001-A-1 colonies had normal stem cell morphology with discrete and well-defined boundaries and a high nucleus to cytoplasm ratio (Fig. 1C). Immunofluorescent staining confirmed the expression of nuclear pluripotency markers, OCT4 and NANOG (Fig. 1D) and flow cytometry showed that > 92% of the cell population co-expressed pluripotency markers SSEA-3 and TRA-1-81 and 98% co-expressed CD9 and TRA-1-81 (Fig. 1E). The ability of this iPSC line to differentiate into the three main germ layers was confirmed. Directed differentiation to definitive endoderm (Loh et al., 2014) induced co-expression of the endoderm markers SOX17 (Fig. 1F), CXCR4 and EpCAM (Fig. 1F). Neuroectoderm was demonstrated by expression of Nestin and PAX6 (Fig. 1G) after directed differentiation (Tchieu et al., 2017). CD45 expression (Fig. 1H) indicated MCRIi001-A-1 could differentiate into blood, a mesoderm derivative (Ng et al., 2016). Differentiation to sclerotome (Loh et al., 2016), with consequent dramatically increased mRNA expression of the marker genes, MEOX1 and FOXC2 (Fig. 1H) provided further confirmation of mesoderm differentiation. Comparison with MCRIi001-A showed similar sclerotome differentiation of the OI mutant iPSCs and the isogenic parental line Table 1.

Materials and methods

Cell culture

The human iPSC lines were cultured at 37 °C with 5% CO_2 on mitotically inactivated mouse embryonic fibroblasts (MEFs) in KnockOut DMEM/F-12 with 20% KnockOut Serum Replacement, 2 mM GlutaMax, 1% Non-Essential Amino Acid Solution, 0.1 mM β-mercaptoethanol (all from Thermo Fisher Scientific) and 50 ng/ml FGF2 (PeproTech) (iPSC media). Media was changed daily and cells were passaged (1:6 split) every 3 days with 0.5 mM EDTA in PBS.

Cas9-Gem-mediated gene editing

The pDNR-SPCas9-Gem expression vector (Howden et al., 2016) (a gift from Sara Howden & Melissa Little, Addgene plasmid # 80424) was linearized with *PmeI* and transcribed *in vitro* using the mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Thermo Fisher Scientific) according the manufacturer's instructions to generate the capped and polyadenylated mRNA. The sgRNA was designed using *E*-CRISP version 5.3 (http://www.e-crisp.org/E-CRISP), annealed and ligated into pSMART-sgRNA (Howden et al., 2016) (a gift from Sara Howden & Melissa Little, Addgene plasmid # 80427). The double-stranded oligonucleotide repair template (1000 bp gBlocks, Integrated DNA Technologies) containing the OI26 mutation and eight synonymous changes (Fig. 1B) was cloned into pUC19 and sequenced to confirm that only the desired sequence changes were present. For electroporation, 80% confluent hiPSCs were dissociated into single cells with TryPLE Express (Thermo Fisher Scientific) and ~1×10^6 cells were transfected with 5 µg Cas9-Gem mRNA, 2 µg sgRNA, 5 µg dsODN and 0.2 µg pEFBOS-GFP using the Neon® Transfection kit (Thermo Fisher Scientific) as previously published (Howden et al., 2016). Electroporated cells were immediately plated onto MEF-coated culture dishes in iPSC media containing 10 µM Rock inhibitor Y-27632 (StemCell Technologies) for the first 24 h. On the third day, GFP-positive single cells were flow sorted (FACSAria Fusion Cell Sorter, BD Biosciences) into 96 well plates plated with MEFs in iPSC medium. Single cell clones were expanded to confluency and a fraction of each colony collected for PCR screening and DNA sequencing.

Allele specific sequencing

Genomic PCR products (for primers see Table 2) were cloned using a TOPO TA Cloning Kit (Thermo Fisher Scientific) and sequenced using M13 forward and reverse primers.

Immunocytochemistry

Cells in 12 well culture plates or on glass slides were fixed in 4% paraformaldehyde for 20 min at room temperature, followed by permeabilization with solution containing 0.05% Triton X-100 in PBS for 10 min at 4 °C. The cells were then incubated in blocking solution consisting of 3% bovine serum albumin in 0.1% PBST (PBS + Tween-20) for 30 min at room temperature and stained overnight at 4 °C with primary antibodies diluted in 1% bovine serum albumin in 0.1% PBST. Secondary antibodies, diluted in 1% bovine serum albumin in 0.1% PBST, were applied for 1 h at room temperature. Antibodies are listed in Table 2. Cells were incubated with DAPI (1 µg/ml) to counterstain nuclei prior to visualisation by fluorescent microscopy (Olympus IX70).

Flow cytometry analysis

Cells were dissociated with TrypLE (Thermo Fisher Scientific) for 3 min at 37 °C. Harvested iPSCs were incubated with directly conjugated antibodies (Table 2) diluted in PBS containing 3% fetal bovine serum (GE Healthcare) for 20 min at 4 °C. Isotype controls were used for gating (Table 2). Propidium iodide (Sigma) was used to identify dead cells. Samples were analyzed using a BD LSRII (BD Biosciences) using BD FACSDiva software.

Differentiation

iPSCs were differentiated in monolayer culture into definitive endoderm (Loh et al., 2014) and analyzed by flow cytometry and immunocytochemistry after 4 days. Ectoderm potential was assessed by

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency marker	Mouse anti-CD9 Monoclonal Antibody, FITC conjugated, Clone M-L13	1/20	BD Biosciences Cat# 555371, RRID: AB_395773
Pluripotency marker	Alex Fluor 647 anti-human TRA-1-81 antibody	1/50	BioLegend Cat# 330706, RRID: AB_1089242
Pluripotency marker	Mouse anti-Human NANOG purified, eBioscience	1/200	ThermoFisher Scientific Cat# 14–5769-80, RRID: AB_467573
Pluripotency marker	Oct-4A (C30A3) Rabbit mAb	1/400	Cell Signaling Technology Cat# 2840, RRID: AB_2167691
Differentiation marker	PE/Cy7 conjugated anti-human CD184 (CXCR4) antibody	1/40	BioLegend Cat# 306514, RRID: AB_2089651
Differentiation marker	Alex Fluor 488 anti-human/mouse SSEA-3 antibody	1/30	BioLegend Cat# 324222, RRID: AB_2561506
Differentiation marker	BV 421 conjugated anti-human CD326 (EPCAM) antibody	1/50	BioLegend Cat# 324220, RRID: AB_2563847
Differentiation marker	BV 421 anti-human CD45 antibody	1/40	BioLegend Cat# 304032, RRID: AB_2561357
Differentiation marker	Goat anti-SOX17	1/500	R&D Systems Cat# AF1924, RRID: AB_355060
Differentiation marker	Mouse Anti-Nestin Antibody, clone 10C2	1/300	Merck Cat# MAB5326, RRID: AB_2251134
Differentiation marker	Mouse anti-PAX6	1/300	DSHB Cat# Pax6, RRID: AB_2315070
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	Donkey anti-Goat IgG (H&L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	1/1000	ThermoFisher Scientific Cat# A21447, RRID: AB_2535864
Secondary antibody	Goat anti-Rabbit IgG (H&L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1/1000	ThermoFisher Scientific Cat# A11008, RRID: AB_143165
Isotype control	FITC Mouse IgG1κ	1/100	BD Biosciences Cat# 555748, RRID: AB_396090
Isotype control	APC Mouse IgG1κ	1/100	BD Biosciences Cat# 555751, RRID: AB_398613
Isotype control	Brilliant Violet 421 Mouse IgG2bκ	1/100	BioLegend Cat# 400341, RRID: AB_10898160
Isotype control	PE/Cy7 Mouse IgG1κ	1/100	BioLegend Cat# 400126, RRID: AB_326448

Primers		
	Target	Forward/Reverse primer (5′-3′)
COL1A1-exon49-sgRNA	*COL1A1* exon 49	CACCGACAAGAGGCATGTCTGGTT/AAACAACCAGACATGCCTCTTGTC
Mutant allele specific PCR and sequencing	*COL1A1* exon 48–49,537 bp	CTGGTTTCGACTTCAGCTTCCTG/CTCGTCTGACTAAACAGTGTCGT
Wild-type allele specific PCR and sequencing	*COL1A1* exon 48–49 537 bp	CTGGTTTCGACTTCAGCTTCCTG/TCTCGCCGAACCAGACATGCCTC
PCR for cloning and sequencing edited region	*COL1A1* exon 48–50 1050 bp	CTGGTTTCGACTTCAGCTTCCTG/TCGGCGCGGATCTCGATCTCGTT
Sclerotome marker	*MEOX1*	ACTCGGCTCCGCAGATATGA/GAACTTGGAGAGGCTGTGGA
Sclerotome marker	*FOXC2*	TGGTATCTCAACCACAGCGG/CCCGGGACACGTCAGTATTT
Housekeeping gene	*ACTB*	AAGTCCCTTGCCATCCTAAAA/ATGCTATCACCTCCCCTGTG

differentiation to neuroectoderm for 12 days (Tchieu et al., 2017) and immunocytochemical analysis. Mesodermal differentiation potential was confirmed by directed differentiation to blood mesoderm for 22 days (Ng et al., 2016) and sclerotome over 6 days (Loh et al., 2016) and analysis by flow cytometry and quantitative RT-PCR.

PCR

Polymerase chain reaction (PCR) was carried out in a 20 µl reaction containing 1× GoTaq reaction buffer (Promega), 1.5 mM $MgCl_2$ (Promega), 250 µM dNTPs (Roche), 0.05 U GoTaq G2 Hot Start polymerase (Promega), 5 ng forward and reverse primers and up to 50 ng DNA template. PCR was performed using an Applied Biosystems (Veriti 96) thermocycler. Touchdown PCR conditions were 95 °C for 1 min, 14 cycles of 95 °C for 20 s, 20 s annealing at 63–56 °C (decreasing by 0.5 °C each cycle), and 72 °C for 20 s, then 16 cycles of 95 °C for 20 s, 56 °C for 20 s, and 72 °C for 20 s, followed by a final extension at 72 °C for 7 min. PCR products were analyzed by agarose gel electrophoresis.

Quantitative RT-PCR

RNA was extracted from cells using TRIzol (Invitrogen, 1 ml TRIzol per 10 cm^2 culture dish). 1 µg of mRNA was used for cDNA synthesis by QuantiTect® Reverse Transcription Kit (Qiagen). qPCR was performed using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies) in a 10 µl reactions per well in a 384-well plate containing 5 µl 2× SYBR Green Master Mix, 1 µl cDNA (~ 200 ng of cDNA) and 4 µl of 1 µM primer stock (forward and reverse primers). Thermal cycling was conducted on LightCycler® 480 II (Roche). Serial dilutions of cDNA were used to produce a standard curve for each reference and target gene.

SNP analysis

Cell pellets (at passage 2) were provided to the Victorian Clinical Genetics Service (Murdoch Children's Research Institute, Melbourne, Australia) and genomic DNA was analyzed using an Infinium CoreExome-24 v1.1 SNP array (Illumina). MCRIi001-A-1 was compared to the parental line, MCRIi001-A (PB001.1) using SNPduo (http://pevsnerlab.kennedykrieger.org/SNPduo/).

Mycoplasma detection

Absence of mycoplasma contamination was confirmed by PCR by the commercial service provider Cerberus Sciences (Adelaide, Australia).

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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.2019.101449.

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