

Lab resource: Stem Cell Line

Generation of a heterozygous *COL2A1* (p.R989C) spondyloepiphyseal dysplasia congenita mutation iPSC line, MCRIi001-B, using CRISPR/Cas9 gene editing



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ABSTRACT

To produce an *in vitro* model of the human chondrodysplasia, spondyloepiphyseal dysplasia congenita, we used CRISPR/Cas9 gene editing to generate a heterozygous patient *COL2A1* mutation in an established control human iPSC line. The gene-edited heterozygous *COL2A1* p.R989C line had a normal karyotype, expressed pluripotency markers, and could differentiate into cells representative of the three embryonic germ layers. When differentiated into cartilage this cell line and the parental isogenic control may be used to explore disease mechanisms and evaluate therapeutic approaches.

Resource table

Unique stem cell line identifier	MCRIi001-B
Alternative name(s) of stem cell line	MCRIi001-A-SOX9tdTom-COL2A1p.R989C; PB001-SOX9tdTom-COL2A1p.R989C
Institution	Murdoch Children's Research Institute, Melbourne, Australia
Contact information of distributor	Associate Professor Shireen Lamandé shireen.lamande@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	Spondyloepiphyseal dysplasia; OMIM #183900
Gene/locus	<i>COL2A1</i> c.2965C>T Chromosome 12q13.11
Method of modification	CRISPR/Cas9
Name of transgene or resistance	N/A

Resource table (continued)

Unique stem cell line identifier	MCRIi001-B
Inducible/constitutive system	N/A
Date archived/stock date	January 2019
Cell line repository/bank	https://hpscreg.eu/cell-line/MCRIi001-B
Ethical approval	RCH Human Research Ethics Committee 35121A; MCRI Animal Ethics Committee A788

1. Resource utility

This heterozygous *COL2A1* p.R989C human iPSC line, together with its isogenic control line, provide an experimental model to explore spondyloepiphyseal dysplasia congenita disease mechanisms in human cartilage *in vitro* and a platform for testing new therapeutic approaches.

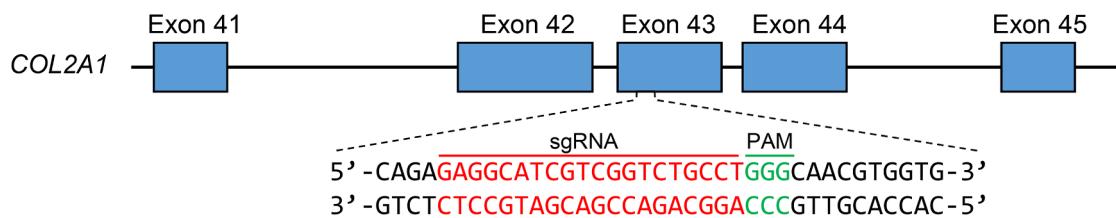
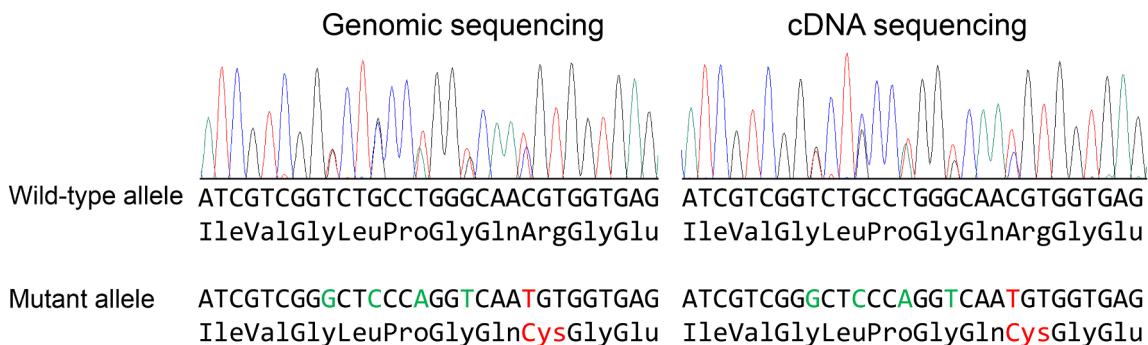
2. Resource details

Genetic cartilage disorders are a significant human disease burden and although many mutations have been defined, our understanding of the cellular pathological mechanisms is incomplete. Mutations in the

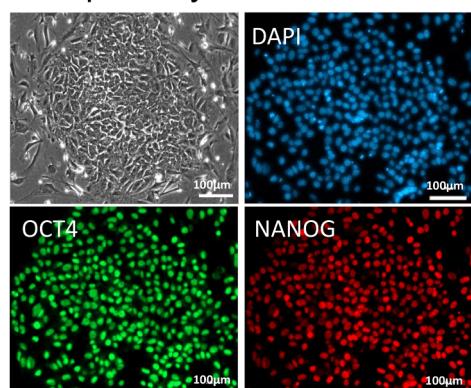
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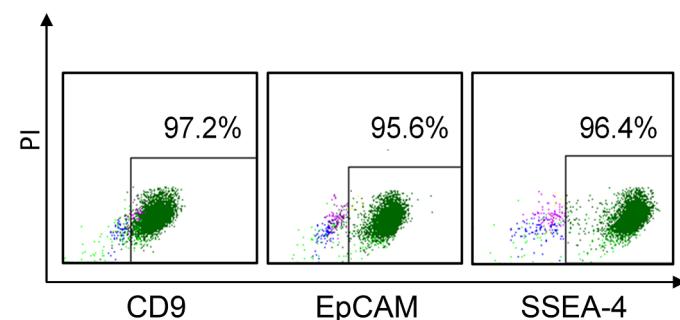
A Gene editing- *COL2A1* p.R989C

**B**

C Pluripotency



D Pluripotency



E Teratoma - Differentiation potential

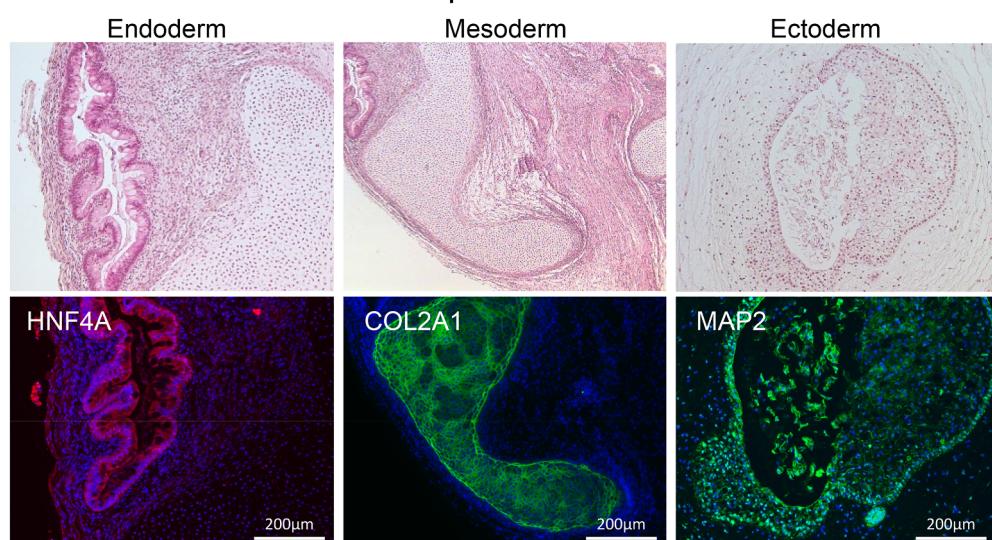


Fig. 1. Cellular and molecular characterization.

gene for collagen II, the major structural protein of the cartilage extracellular matrix, are common, causing a spectrum of mild to severe and perinatal lethal skeletal phenotypes (<https://databases.lovd.nl/shared/genes/COL2A1>). In this study, we used CRISPR/Cas9 gene editing to generate a human iPSC line with a recurrent heterozygous *COL2A1* mutation (*COL2A1* exon 43c.2965C>T; p.R989C) causing spondyloepiphyseal dysplasia congenita (SEDC; OMIM #183900) (Chan et al., 1993). In transfected cells the heterozygous *COL2A1* p.R989C mutation destabilizes the structure of the collagen triple helix, resulting in the intracellular retention of the misfolded collagen and the possible initiation of a pathological cellular unfolded protein response (Hintze et al., 2008). This mutant line and the parental isogenic control line will be used to produce *in vitro* human cartilage disease models which are critical to understand disease mechanisms in the context of human chondrocytes and to test new therapeutic approaches *in vitro*.

A control human iPSC line, MCRIi001-A-2 which contains a SOX9-tdTomato reporter to monitor cartilage differentiation (Nur Patria et al., 2020) was co-transfected with pSpCas9(BB)-2A-GFP encoding an sgRNA (Fig. 1A) and a 900 bp repair template spanning *COL2A1* exons 42–44. In addition to the patient-specific *COL2A1* mutation, the repair template contained synonymous base pair changes (Fig. 1B, shown in green) to prevent CRISPR-Cas9 re-cutting the gene edited allele and to facilitate PCR screening. After sorting for GFP positive cells, heterozygous gene edited clones were identified by PCR using wild-type and mutant specific primer sets (Table 2). Sequencing confirmed the heterozygous *COL2A1* c.2965C>T mutation in the genomic DNA and the mRNA in clone MCRIi001-B (Fig. 1B).

MCRIi001-B had normal stem cell morphology, observed by formation of compact colonies with well-defined boundaries and a high nucleus to cytoplasm ratio (Fig. 1C). Immunofluorescent staining confirmed the expression of pluripotency marker genes, OCT4 and NANOG (Fig. 1C) and flow cytometry showed that more than 95% of the cell population expressed pluripotency markers, SSEA-3, CD9 and EPCAM (Fig. 1D). The ability of the gene-edited line to differentiate into the three embryonic germ layers was confirmed in teratomas formed by injecting the iPSCs into immunodeficient mice. Haematoxylin and eosin (H&E) staining revealed structures consistent with the three germ layers. Intestinal epithelium-like tissue (endoderm) was identified by the characteristic morphology and by hepatocyte nuclear factor 4 alpha, HNF4A, immunostaining (Fig. 1E). Mesoderm was represented by cartilaginous tissues (Fig. 1E) expressing collagen II (*COL2A1*) and ectoderm by the presence of squamous epithelium which stained positive for microtubule associated protein 2, MAP2 (Fig. 1E). SNP array analysis demonstrated that there were no aneuploidies or large deletions or insertions, however this does not preclude the presence of balanced translocations. SNP Duo analysis confirmed that MCRIi001-B had >99.9% identity of with the parental line MCRIi001-A-2 (Table 1),

(Supplementary Fig. 1). MCRIi001-B was free from mycoplasma contamination (Table 1, Supplementary Fig. 2, Sample Ref # 03-2017-273).

3. Materials and methods

3.1. Cell culture

The human iPSCs were cultured at 37 °C, 5% CO₂ on mitotically arrested mouse embryonic fibroblasts (MEFs) in DMEM/F-12 supplemented with 20% Knock Out Serum Replacement, 1% Non-Essential Amino Acids, 2 mM GlutaMAX, and 50 µM 2-mercaptoethanol (all from Thermo Fisher), and 50 ng/mL fibroblast growth factor 2 (PeproTech) (iPSC media). The medium was changed daily and cells were passaged (1:4–1:6) every 3 days with 0.5 mM EDTA in PBS (Thermo Fisher).

3.2. CRISPR/Cas9-mediated gene editing

The sgRNA to target *COL2A1* was designed using a CRISPR design tool (<http://crispr.mit.edu/>). The sgRNA oligonucleotides were annealed and ligated into pSpCas9(BB)-2A-GFP (PX458) (Addgene # 48138). The two complementary repair template oligonucleotides containing the *COL2A1* mutation and four synonymous changes (Integrated DNA Technologies) were annealed and ligated into pUC19 (NEB #N3041S). The pSpCas9-sgRNA and pUC19-repair template plasmids were sequenced to confirm that errors had not been introduced. For electroporation, 1 × 10⁶ cells were transfected with 2 µg sgRNA-pSpCas9-GFP plasmid and 5 µg repair template plasmid using the Neon® Transfection kit (Thermo Fisher). Electroporated cells were 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 electroporated cells were sorted into 96 well plates pre-plated with MEFs (BD InFlux cell sorter, BD Biosciences). Single cell clones were expanded and screened by PCR and sequencing.

3.3. PCR for screening and sequencing

Genomic DNA was extracted using a DNAeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. Wild-type and mutant allele specific PCRs were used to identify gene-edited iPSCs using GoTaq Hot Start polymerase (Promega) (for primers see Table 2). Touchdown PCR conditions were; 95 °C for 2 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 45 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. Clones positive for wild-type and mutant allele specific PCRs were further screened for correct gene-editing by PCR and sequencing using the BigDye™

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 C
	Quantitative analysis (Flow cytometry)	CD9: 97.2% EPCAM: 95.6% SSEA-4: 96.4%	Fig. 1 panel D
Genotype	SNP array (resolution 0.5 Mb)	46XY No aneuploidies were detected	Supplementary Fig. 1
Identity	Genetic analysis	SNPduo of SNParrys to compare parental and gene edited clones. Identical genotypes (>99.9%) for the entire genome, indicating the lines are from the same individual	Supplementary Fig. 1
Mutation analysis	Sequencing	Heterozygous <i>COL2A1</i> p.R989C allele gene edited confirmed	Fig. 1 panel B
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR. Negative	Supplementary Fig. 2
Differentiation potential	Teratoma formation	Endoderm: HNF4A Mesoderm: COL2A1 Ectoderm: MAP2	Fig. 1 panel E

Table 2
Reagent details.

Antibodies used for immunocytochemistry/flow cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency marker (flow cytometry)	BV 421 conjugated anti-human CD326 (EPCAM) antibody	1/50	BioLegend Cat# 324220, RRID: AB_2563847
Pluripotency marker (flow cytometry)	Mouse anti-CD9 Monoclonal Antibody, FITC conjugated, Clone M-L13	1/20	BD Biosciences Cat# 555371, RRID: AB_395773
Pluripotency marker (flow cytometry)	Alexa Fluor 647 anti-human SSEA-4 antibody	1/40	BioLegend Cat# 330408, RRID: AB_1089200
Pluripotency marker (immunohistochemistry)	Anti-Human Nanog Monoclonal Antibody, clone hNanog.1	1/200	Thermo Fisher Cat# 14-5769-80, RRID: AB_467573
Pluripotency marker (immunohistochemistry)	Oct-4A (C30A3) Rabbit mAb	1/400	Cell Signaling Technology Cat# 2840, RRID: AB_2167691
Differentiation marker (immunohistochemistry)	Anti-Collagen Type II Antibody, clone 6B3 (COL2A1)	1/150	Merck Cat# MAB8887, RRID: AB_2260779
Differentiation marker (immunohistochemistry)	HNF4A Monoclonal Antibody (K9218)	1/300	Thermo Fisher Cat# MA1-199, RRID: AB_2633309
Differentiation marker (immunohistochemistry)	Monoclonal Anti-MAP2 (2a + 2b) antibody	1/250	Merck Cat# M1406, RRID: AB_477171
Differentiation marker (immunoblotting)	Anti-Sox9 Antibody	1/2000	Millipore Cat# AB5535, RRID: AB_2239761
Secondary antibody	Donkey anti-Mouse IgG (H&L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	1/1000	Thermo Fisher Cat# A21203, RRID: AB_2535789
Secondary antibody	Goat anti-Rabbit IgG (H&L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1/1000	Thermo Fisher Cat# A11008, RRID: AB_143165
Secondary antibody	Goat anti-Mouse IgG (H&L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1/500	Thermo Fisher Cat# A11029, RRID: AB_2534088
Isotype control	BV 421 Mouse IgG2bk	1/100	BioLegend Cat# 400341, RRID: AB_10898160
Isotype control	FITC Mouse IgG1x	1/100	BD Biosciences Cat# 555748, RRID: AB_396090
Isotype control	Alexa Fluor 647 Mouse IgM, κ	1/100	BioLegend Cat# 401618; RRID: AB_2802167
Primers			
	Target	Forward/Reverse primer (5'-3')	
COL2A1-exon 43-sgRNA	COL2A1 exon 43	CACCGAGGCATCGTCGGTCTGCCT/ AAACAGGCAGACCGGACGGATGCCCT	
Mutant allele specific PCR	COL2A1 exon 42–43, 448 bp	TTCTCCTTCTAGGGCAACCC/CACCACATTGACCTGGGAGC	
Wild type allele specific PCR	COL2A1 exon 43–44, 608 bp	GTCGGTCTGCCTGGCAACGTGG/ CCTCTCCTCTTGCCCCACTC	
PCR for sequencing	COL2A1 exon 42–44, 1215 bp	CCAGAGCTGTCTCACATGGTGA/CCTCTCCTCTTGCCCCACTC	
Primer for sequencing edited genomic region	COL2A1 exon 42	CTCAGAGCATGGGTAGGAG	
RT-PCR and sequencing	COL2A1 c.2334–2851	CAAGGGTGAGCAAGGAGAGG/CAGGAGGACCTCTGTCTCCA	

Terminator v3.1 cycle sequencing kit (Thermo Fisher). RNA was extracted using Trizol (Invitrogen) and 500 ng was used for cDNA synthesis (20 µl reaction) using a QuantiTect® Reverse Transcription Kit (Qiagen). The cDNA was diluted 1:5 and 1 µl used for PCR and sequencing as above.

3.4. Flow cytometry analysis

Cells were dissociated using TrypLE (Thermo Fisher) and large cell clumps removed using a FACS tube strainer (Corning). Single-cell suspensions were incubated with directly conjugated antibodies (Table 2) diluted in PBS containing 2% fetal bovine serum (GE Healthcare) for 20 min at 4 °C. Isotype controls were used to set negative gates (Table 2). Propidium iodide (Sigma) was used to identify dead cells. Samples were analyzed using a BD LSRII (BD Biosciences) using BD FACSDiva and FCS Express software.

3.5. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 20 min at room temperature, then permeabilized with 0.05% Triton X-100 in PBS for 10 min. Samples were blocked in PBST (PBS + 0.1% Tween-20) containing 3% bovine serum albumin (BSA) for 30 min and stained with primary antibodies diluted in PBST containing 1% BSA at 4 °C, overnight. Secondary antibodies were applied for 1 hr at room temperature. Nuclei were stained with DAPI (1 µg/ml) and cells visualised by Observer Z.1 microscope (Zeiss). Antibodies and their working dilutions are listed in Table 2.

3.6. In vivo differentiation (teratoma formation) and analysis

Teratomas were generated in immunodeficient mice as described (International Stem Cell Initiative, 2018). Teratomas were harvested 8–12 weeks post injection. Teratoma tissues were fixed in 1X Confix (Australian Biostain) and paraffin-embedded. Sections (5 µm) were stained with H&E or specific antibodies as above.

3.7. SNP analysis

Genomic DNA was isolated from MCRIi001-B cells at passage 9 and analyzed (Victorian Clinical Genetics Service, Murdoch Children's Research Institute, Australia) using an Infinium CoreExome-24 v1.1 SNP array (Illumina). MCRIi001-B was compared to the parental line, MCRIi001-A-2 using SNPduo (<http://pevnsnerlab.kennedykrieger.org/SNPduo/>).

3.8. Mycoplasma detection

hiPSCs were tested for mycoplasma using PCR, performed 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.

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.2020.101843>.

References

- Chan, D., Taylor, T.K., Cole, W.G., 1993. Characterization of an arginine 789 to cysteine substitution in alpha 1 (II) collagen chains of a patient with spondyloepiphyseal dysplasia. *J. Biol. Chem.* 268, 15238–15245.
- Hintze, V., Steplewski, A., Ito, H., Jensen, D.A., Rodeck, U., Fertala, A., 2008. Cells expressing partially unfolded R789C/p.R989C type II procollagen mutant associated with spondyloepiphyseal dysplasia undergo apoptosis. *Human Mutat.* 29, 841–851.
- International Stem Cell Initiative, 2018. Assessment of established techniques to determine developmental and malignant potential of human pluripotent stem cells. *Nat. Commun.* 9, 1925.
- Nur Patria, Y., Lilianty, J., Elefanti, A.G., Stanley, E.G., Labonne, T., Bateman, J.F., Lamande, S.R., 2020. Generation of a SOX9-tdTomato reporter human iPSC line, MCRIi001-A-2, using CRISPR/Cas9 editing. *Stem Cell Res.* 42, 101689.