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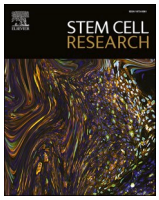

Lab Resource: Single Cell Line

Using CRISPR/Cas9 to generate a heterozygous *COL2A1* p.R719C iPSC line (MCRIi019-A-6) model of human precocious osteoarthritis

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A B S T R A C T

The human iPSC line MCRIi019-A-6 was generated using CRISPR/Cas9-mediated gene editing to introduce a heterozygous *COL2A1* exon 33 c.2155C>T (p.R719C) mutation into the control human iPSC line MCRIi019-A. Both the edited and parental lines display typical iPSC characteristics, including the expression of pluripotency markers, the ability to be differentiated into the three germ lines, and a normal karyotype. This cell line, along with the isogenic control line, can be used to study the molecular pathology of precocious osteoarthritis in a human model, more broadly understand type II collagenopathies, and explore novel therapeutic targets for this class of diseases.

Resource table

Unique stem cell line identifier	MCRIi019-A-6
Alternative name(s) of stem cell line	1502.3 *COL2A1* p.R719C (MCRIi019-A-6)
Institution	Murdoch Children's Research Institute, Melbourne, Australia
Contact information of the reported cell line distributor	Professor Matthew Shoulders mshoulde@mit.edu Associate Professor Shireen Lamandé shireen.lamande@mcri.edu.au
Type of cell line	iPSC
Origin	Human
Additional origin info *(applicable for human ESC or iPSC)*	Age: 12 weeks gestation Sex: Female Ethnicity: Black
Cell Source	Dermal fibroblast-derived human induced pluripotent cell line MCRIAi019-A (https://hpscreg.eu/cell-line/MCRIAi019-A)
Method of reprogramming	Episomal vectors
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	N/A
The cell culture system used	Matrigel (Corning)
Type of the Genetic Modification	Induced mutation
Associated disease	Osteoarthritis with Mild Chondrodysplasia (OMIM #604864)
Gene/locus	*COL2A1* c.2155 CGT>TGT (p.R719C) Chromosome 12q13.11
Method of modification / user-customisable nuclease (UCN) used, the resource used for design optimisation	CRISPR/Cas9
User-customisable nuclease (UCN) delivery method	Plasmid transfection
All double-stranded DNA genetic material molecules introduced into the cells	pSMART-COL2A1-sgRNA
Analysis of the nuclease-targeted allele status	Sequencing of the targeted allele
Method of the off-target nuclease activity prediction and surveillance	SNP array and STR profiling
Descriptive name of the transgene	N/A
Eukaryotic selective agent resistance cassettes (including inducible, gene/cell type-specific)	N/A

(*continued on next page*)

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Resource table (*continued*)

Inducible/constitutive expression system details	N/A
Date archived/stock creation date	February 2020
Cell line repository/bank	https://hpscreg.eu/cell-line/MCRIi019-A-6
Ethical/GMO work approvals	This study was approved through the Human Research Ethics Committee of the Royal Children's Hospital (HREC35121A), Victoria, Australia.
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	pSMART-sgRNA (Sp) was a gift from Sara Howden & Melissa Little (Addgene plasmid # 80427; https://n2t.net/addgene:80427; RRID:Addgene_80427)

1. Resource utility

This precocious osteoarthritis iPSC line (*COL2A1* p.R719C) and its parental isogenic control provide human cell models to study precocious osteoarthritis and type II collagenopathies. These cell lines facilitate experiments to elucidate underlying mechanisms of cellular pathology, as well as efforts to discover new therapeutic approaches for this class of diseases (See Table 1).

2. Resource details

Collagen type II constitutes the main proteinaceous scaffold of the cartilage extracellular matrix. The mature protein is composed of a long triple-helical domain made up of repeated Gly-Xaa-Yaa triplets. While Gly substitutions destabilize the triple helix (Wong and Shoulders, 2019), the biophysical consequences of Xaa-or Yaa-position substitutions for collagen-II triple helices are less clear (Chakkalakal et al., 2018).

Autosomal dominant mutations in the gene encoding type II procollagen (*COL2A1*) lead to a variety of disorders with symptoms ranging from mild to severe (https://databases.lovd.nl/shared/genes/COL2A1). These disorders are known as type II collagenopathies. Patients commonly display skeletal phenotypes, including aberrant development of the growth plate and articular cartilage defects. One such disease is precocious osteoarthritis arising from the heterozygous *COL2A1* c.2155 CGT>TGT in exon 33 encoding p.R719C, an arginine to cysteine substitution in the Yaa position (Knowlton et al., 1990). Patients with this mutation present with signs of osteoarthritis, including pain and progressive articular cartilage degeneration, starting as early as the second decade of life (OMIM #604864).

Collectively, collagenopathies are difficult to study owing to a paucity of robust model systems. They are poorly modeled in cells alone, as pathology is most acutely observed in affected tissues as a whole. They are also often poorly represented by mouse models, owing to differences in physiology and pathology (Perlman, 2016). We developed this human iPSC mutant line, along with its parental isogenic control line, to provide an *in vitro* human tissue system to model precocious osteoarthritis, to help uncover the molecular basis of pathology, and to discover new therapeutic targets for type II collagenopathies.

The *COL2A1* p.R719C human iPSC line MCRIi019-A-6 described herein was produced by CRISPR/Cas9 editing of a previously characterized control iPSC line, MCRIi019-A (Kung et al., 2020), derived from dermal fibroblasts (ATCC cat: CRL-1502; https://hpscreg.eu/cell-line/MCRIAi019-A). Briefly, the control line was co-transfected with

Table 1
Characterization and validation.

Classification (optional *italicized*)	Test	Result	Data
Morphology	Photography	Typical primed pluripotent human stem cell morphology	Fig. 1 panel C
Pluripotency status evidence for the described cell line	Qualitative analysis *(i.e. Immunocytochemistry, western blotting)*	Expression of pluripotency markers Oct4 and Nanog	Fig. 1 panel C
	Quantitative analysis *(i.e. Flow cytometry, RT-qPCR)*	N/A	N/A
Karyotype	Karyotype (G-banding) and higher-resolution, array-based assays (KaryoStat, SNP, etc.)	46XX, Resolution: 0.50 Mb 20 metaphase cells counted SNP array	Fig. 1 panel G Supplementary Fig. 1
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site or targeted allele-specific PCR	Heterozygous *COL2A1* c.2155C>T mutation confirmed in MCRIi019-A-6	Fig. 1 panel B
	Evaluation of the - (homo-/hetero-/hemi-) zygous status of introduced genomic alteration (s)	PCR and subsequent NGS sequencing confirm the presence of both parental and edited alleles	Fig. 1 panel B
	Transgene-specific PCR (when applicable)	N/A	N/A
Verification of the absence of random plasmid integration events	PCR	Plasmid not detected in gDNA of gene-edited cell line	Supplementary Fig. 4
Parental and modified cell line genetic identity evidence	STR analysis, microsatellite PCR (mPCR) or specific (mutant) allele seq	DNA STR Profiles match	Supplementary Fig. 2
		Amelogenin + 23 loci are matched between cell lines	Supplementary Fig. 2
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR or RT-PCR product)	Heterozygous *COL2A1* c.2155C>T mutation confirmed in MCRIi019-A-6 in gDNA and cDNA, and not in the parental MCRIi019-A	Fig. 1 panel B
	PCR-based analyses	N/A	N/A
	Southern Blot or WGS; western blotting (for knock-outs, KOs)	N/A	N/A
Off-target nuclease activity analysis	PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing	N/A	N/A
Specific pathogen-free status	Mycoplasma	Mycoplasma testing by luminescence. Both lines confirmed negative.	Supplementary Fig. 3
Multilineage differentiation potential	Directed differentiation	Endoderm: Sox17 Ectoderm: Nestin and Pax6 Mesoderm: Brachyury	Fig. 1 panels D, E, F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

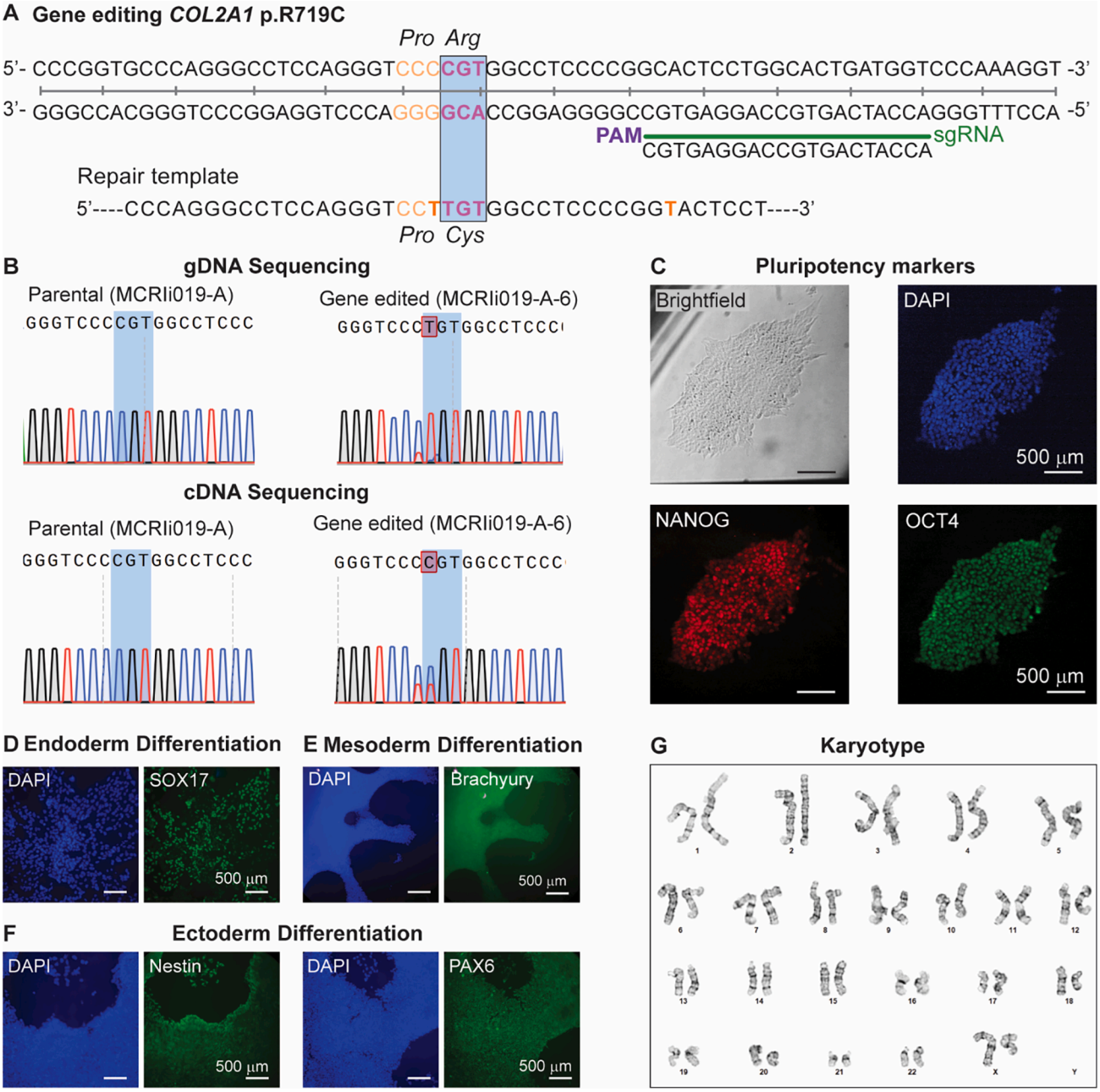

Fig. 1.

Cas9gem mRNA, a plasmid encoding a *COL2A1*-specific sgRNA, and an oligodeoxynucleotide (ODN) repair template containing the desired *COL2A1* mutation with homology arms flanking the targeted nucleotide (Fig. 1A). Targeted clones were identified by PCR, and the heterozygous mutation was confirmed by next generation sequencing (NGS) of the gDNA and mRNA (Fig. 1B).

The gene edited line displayed normal stem cell morphology (Fig. 1C, *brightfield*) and expression of pluripotency markers Oct4 and Nanog, as observed by immunocytochemistry (Fig. 1C). Moreover, the cell line could be differentiated into the three main germ layers: endoderm, confirmed by the expression of endoderm marker Sox17 (Fig. 1D); mesoderm, confirmed by the expression of Brachyury (Fig. 1E); and neuroectoderm, confirmed by the co-expression of Nestin and Pax6 (Fig. 1F).

G-Band karyotyping confirmed the absence of chromosomal abnormalities in the edited iPSC line (Fig. 1G). Single nucleotide polymorphism (SNP) arrays revealed no aneuploidies or large deletions or insertions. SNP Duo analysis confirmed that MCRIi019-A-6 has >99.9 % identity to the parental line MCRIi019-A (Kung et al., 2020) (SI, Fig. 1). Short tandem repeat (STR) profiling further confirmed that the alleles of MCRIi019-A-6 match those of the parental line MCRIi019-A, and that both lines were free of other contaminating cell lines (SI, Fig. 2). MCRIi019-A-6 was confirmed free of mycoplasma contamination (SI, Fig. 3).

3. Materials and methods

3.1. Cell culture

MCRIi019-A-6 cells were cultured at 37 °C in 5 % CO_2(g) on Matrigel (Corning)-coated plates in Essential 8 (E8) medium (Thermo Fisher Scientific), which was changed daily. Cells were passaged (1:4–1:6)

Table 2
Reagents details.

Antibodies and stains 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
Ectoderm Marker	PAX6 Monoclonal Antibody (13B10-1A10)	1:200	ThermoFisher Scientific Cat#MA1-109; RRID: AB_2536820
Ectoderm Marker	Anti-Nestin Antibody, clone 10C2	1:200	Merck Cat# MAB5326, RRID: AB_2251134
Mesoderm Marker	Recombinant anti-Brachyury Antibody	1:500	Abcam Cat# ab209665, RRID: AB_2750925
Secondary Antibody	Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:1000	ThermoFisher Scientific Cat# A11008, RRID: AB_ 143165
Secondary Antibody	Donkey anti-Goat IgG H&L (Alexa Fluor® 488)	1:1000	Invitrogen Cat# A-11055, RRID: AB_2534102
Secondary Antibody	Goat anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:500	ThermoFisher Scientific Cat# A11029; RRID: AB_2534088
Site-specific nuclease			
Nuclease information	Cas9	Cas9gem	
Delivery method	Transfection	Neon Transfection System (Thermo Fisher) (1100 V, 30 ms, 1 pulse)	
Selection/enrichment strategy	PCR screening		

Primers and Oligonucleotides used in this study		
	Target	**Forward/Reverse primer (5′-3′)**
sgRNA	*COL2A1* Exon 33	ACCATCAGTGCCAGGAGTGC
Repair Template (ODN) Sequence	*COL2A1* Intron 32 – exon 33/intron 33	CTGCCGCAGGGTGAACGAGGTTTCCCAGGTGAACG
(Mutations bolded, synonymous mutations italicized)		TGGCTCTCCCGGTGCCCAGGGCCTCCAGGGTCC***TT***G TGGCCTCCCCGG***T***ACTCCTGGCACTGATGGTCCCAA AGTAAGTGAGGCTGCATC
COL2A1-719mut – mutation screening PCR (only binds successfully targeted gene)	*COL2A1* Exon 33/intron 34	AGGGCCTCCAGGGTCC**TT**G/ GAAACCTTCATCACCAGGTGC
COL2A1 gDNA – PCR for gDNA sequencing	*COL2A1* Intron 31 - exon 32/intron 34	CTTTGTTCTCCAGGGTGTTCC/ GAAACCTTCATCACCAGGTGC
COL2A1 cDNA – PCR for cDNA sequencing	*COL2A1* Exon 29/exon 35	GCAAAGATGGTGAGACAGGTGCTGCAGGAC/ GGGCTCCCTCAGGGCCTTTCTCAC
pSMART-sgRNA – PCR for off-target integration	pSMART-sgRNA plasmid	ATCGCGTATTTCGTCTCGCT/ CGGACAGGTATCCGGTAAGC

every 3–4 days using 0.5 mM EDTA in PBS for 3–4 min.

3.2. CRISPR/Cas9-mediated gene editing

The sgRNA targeting *COL2A1* was designed using https://crispr.mit.edu/ (in 2018). sgRNA oligonucleotides were annealed, ligated into pSMART-sgRNA plasmid (Addgene #80427), and sequence-confirmed. One million control MCRIi019-A iPS cells were harvested with TrypLE (Thermo Fisher) 2 d after passaging. Cells were electroporated (1100 V, 30 ms, 1 pulse) using Neon Transfection System (Thermo Fisher) with 5 μg of *in vitro*-transcribed Cas9gem mRNA (*In Vitro* Transcription Kit, Takara Bio), 2 μg pSMART-COL2A1-sgRNA plasmid, and 0.5 μg of the ODN repair template incorporating the mutation. Electroporated cells were plated in a Matrigel-coated 6-well dish in E8 medium with 10 μM ROCK inhibitor (Y-27632; Stem Cell Technologies). The next day, media was switched to E8 lacking Y-27632 and changed daily. Individual colonies were isolated and expanded in E8.

3.3. PCR screening and sequencing

Targeted clones were identified using PCR primers that bind only the correctly targeted *COL2A1* R719C mutation. Genomic DNA (gDNA) was extracted using a DNAeasy Kit (Qiagen). PCR was performed using indicated primers in Table 2, with an Applied Biosystems Thermocycler (Veriti), and analyzed via agarose gel electrophoresis.

To further confirm the presence of the mutation in the genome, a PCR product was generated from the gDNA using primers flanking the mutation site (Table 2) and sequenced using Primordium Labs' full plasmid and long PCR product NGS service. To assess the presence of the mutation in mRNA, RNA was extracted using an RNA extraction kit (Omega), reverse transcribed using an Applied Bioscience cDNA kit, amplified using primers flanking the mutation site, and sequenced by Primordium Labs NGS.

To ensure the sgRNA plasmid did not randomly integrate into the genome of the edited line (SI, Fig. 4), PCR was performed using primers specific to the plasmid (Table 2).

3.4. Immunocytochemistry

Cells (passage 37) were fixed in 10 % neutral buffered formalin (Millipore) o/n, followed by permeabilization with 0.05 % Triton X-100 (Millipore) in PBS for 10 min at 4 °C. 3 % bovine serum albumin in PBS for 30 min at rt was used to block non-specific interactions. Cells were then incubated with primary antibodies for 2 h at rt, followed by secondary antibodies and DAPI for 1 h at rt (Table 2). Cells were visualized on a fluorescence microscope (Nikon Eclipse TE200).

3.5. Directed differentiation

iPSCs (passage 39) were differentiated in monolayer culture into the three main germ layers (endoderm, ectoderm, and mesoderm) using the STEMdiff Trilineage Differentiation Kit (Stemcell Technologies). Successful differentiation was assessed by immunocytochemistry for lineage-specific markers (Table 2).

3.6. STR analysis

Live cells were submitted to Cell Line Genetics and genomic DNA was analyzed using GenePrint® 24 System for co-amplification with a five-dye profile of 23 STR loci and Amelogenin (SI, Fig. 2).

3.7. Mycoplasma detection

Cell lines were confirmed free of mycoplasma by a luminescence-based assay (Lonza MycoAlert Plus Mycoplasma Testing Kit) (SI, Fig. 3).

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.2023.103020.

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