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Generation of a heterozygous COL2A1 (p.G1113C) hypochondrogenesis mutation iPSC line, MCRIi019-A-7, using CRISPR/Cas9 gene editing

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A B S T R A C T

The human inherited cartilage disease, Hypochondrogenesis, is caused by mutations in the collagen type II gene, COL2A1. To produce an in vitro disease model, we generated a heterozygous patient mutation, COL2A1 p.G1113C, in an established control human induced pluripotent stem cell (iPSC) line, MCRIi019-A, using CRISPR-Cas9 gene editing. The gene-edited mutant line, MCRIi019-A-7, exhibited normal iPSC characteristics, including normal cell morphology, expression of pluripotency markers, the ability to differentiate into three embryonic germ layers, and normal karyotype. Together with its parental isogenic control, this cell line will be useful for Hypochondrogenesis disease modelling and drug testing.

1. Resource table

Unique stem cell line identifier	MCRIi019-A-7
Alternative name(s) of stem cell line	1502.3 COL2A1 p.G1113C
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	ATCC fibroblast cell line: CRL-1502 Age: 12 weeks gestation Sex: Female Ethnicity: 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	Induced mutation
Associated disease	Achondrogenesis type II/Hypochondrogenesis, OMIM #200610
Gene/locus	COL2A1 c.3397 GGC > TGC (p.G1113C); Chromosome 12q13.11
Method of modification	CRISPR/Cas9 N/A
Name of transgene or resistance	
Inducible/constitutive system	N/A
Date archived/stock date	July 15, 2020
Cell line repository/bank	https://hpscreg.eu/cell-line/MCRIi019-A-7
Ethical approval	Human Research Ethics Committee of the Royal Children's Hospital, Victoria, Australia (HREC 33118)

2. Resource utility

The heterozygous COL2A1 p.G1113C human iPSC line and its isogenic control line provide novel experimental tools to generate cartilage disease models to study Hypochondrogenesis disease mechanisms and test drugs.

3. Resource details

Mutations in COL2A1, the gene encoding the major structural protein of cartilage, cause a spectrum of cartilage disorders varying from mild to severe and perinatal lethal phenotypes (http://databases.lovd.nl/shared/genes/COL2A1). While many mutations have been characterized, the precise molecular mechanisms causing the cellular pathology is unclear, and to develop therapies we need high fidelity human cartilage disease models. Of these COL2A1 disorders, Achondrogenesis II-Hypochondrogenesis (OMIM #200610) is one of the more severe with

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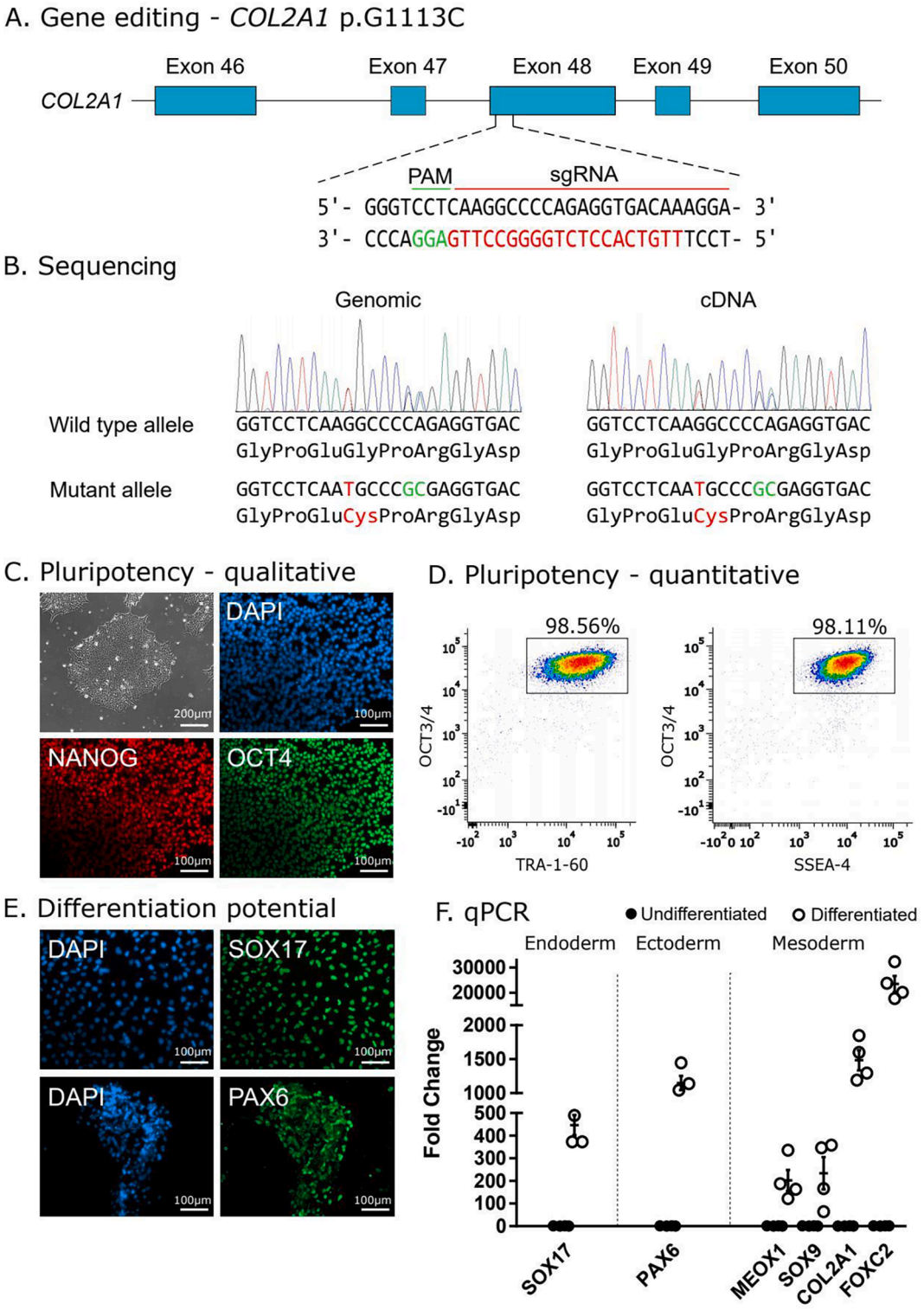

Fig. 1. Cellular and molecular characterization of MCRIi019-A-7.

characteristic features including micromelic dwarfism, macrocephaly, incomplete ossification of the vertebral bodies, and disorganisation of the costochondral junction. It is commonly caused by Gly substitution mutations that interfere with the collagen II protein triple helix integrity. To provide a novel tool to explore the molecular pathology of Hypochondrogenesis, we generated a human iPSC line, MCRIi019-A-7, with a heterozygous COL2A1 c.3397 GGC > TGC; p.G1113C mutation (Mundlos et al., 1996).

Using an established and characterized control human iPSC line, MCRIi019-A (Kung et al., 2020), derived from dermal fibroblasts (ATCC cat: CRL-1502; http://hpscreg.eu/cell-line/MCRIi019-A), the mutation was introduced using CRISPR-Cas9 gene editing. Control iPSCs were co-transfected with Cas9-geminin mRNA, a plasmid encoding a COL2A1-specific sgRNA, and a 122 bp oligodeoxynucleotide (ODN) repair template comprising ~ 60 bp homology arms flanking the mutation (Fig. 1A). The repair template also contained synonymous base pair

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)	OCT3/4 and TRA-1–60: 98.56% OCT3/4 and SSEA-4: 98.11%	Fig. 1 panel D
Genotype	SNP array (resolution 0.5 Mb)	Arr (1–22,X)x2 No aneuploidies were detected	Supplementary Fig. 1
Identity	SNPDuo analysis of SNP array	Identical genotypes (>99.9%) for the entire genome, indicating the lines are from the same individual	Supplementary Fig. 1
Mutation analysis	Sequencing	Heterozygous COL2A1 c.3397 GGC > TGC mutation confirmed in MCRIi019-A-7	Fig. 1 panel B
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR. Negative	Supplementary Fig. 1
Differentiation potential	Directed differentiation	Endoderm: SOX17 Ectoderm: PAX6 Mesoderm: MEOX1, SOX9, COL2A1, FOXC2	Fig. 1panel E and F

changes (Fig. 1B, shown in green) to facilitate PCR screening. Two days post-passaging, individual clones were isolated, expanded, and screened for the introduced mutation by PCR.

The heterozygous COL2A1 c.3397 GGC > TGC mutation was confirmed in clone MCRIi019-A-7 in genomic DNA and mRNA by Sanger sequencing (Fig. 1B). The iPSCs displayed normal stem cell morphology, characterized 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>98% of the cell population expressed pluripotency markers OCT3/4, SSEA4 and TRA-1–60 (Fig. 1D). The iPSCs were differentiated into the three embryonic germ layers by established protocols. Differentiation to endoderm was confirmed by SOX17 staining (Fig. 1E) and qPCR (Fig. 1F); ectoderm by PAX6 staining (Fig. 1E) and qPCR (Fig. 1F), and mesoderm by qPCR of MEOX1, SOX9, COL2A1 and FOXC2) (Fig. 1F). 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 MCRIi019-A-7 had > 99.9% identity of with the parental line MCRIi019-A (Table 1, Supplementary Fig. 1). The cell line was free from mycoplasma contamination (Table 1, Supplementary Fig. 2).

4. Materials and methods

4.1. Cell culture

The human iPSCs were cultured at 37 °C, 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) every 3–4 days with 0.5 mM EDTA in PBS (Thermo Fisher Scientific)

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency marker (immunohistochemistry)	Oct-4A (C30A3) Rabbit Monoclonal Antibody	1:400	Cell Signaling Technology Cat# 2840S, RRID: AB_2167691
Pluripotency marker (immunohistochemistry)	Purified anti-Nanog Antibody	1:400	BioLegend Cat# 674202, RRID: AB_2564574
Pluripotency marker (flow cytometry)	BV421 Mouse Anti-Human TRA-1-60 Antigen	1:20	Becton Dickinson Cat# 562711, RRID: AB_2737738
Pluripotency marker (flow cytometry)	Alexa Fluor 647 anti-human SSEA-4 antibody	1:100	BioLegend Cat# 330408, RRID: AB_1089200
Pluripotency marker (flow cytometry)	PE Mouse anti-OCT3/4	1:5	BD Biosciences Cat# 560186, RRID: AB_1645331
Secondary antibody	Donkey anti-Mouse IgG (H&L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	1:1000	Thermo Fisher Cat# A21203, RRID: AB_141633
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
Primers			
	Target	**Forward/Reverse primer (5'-3')**	
sgRNA	*COL2A1* exon 48	CACCGTTGTCACCTCTGGGGCCTTG / AAACCAAGGCCCCAGAGGTGACAA	
Repair Template (ODN) Mutation in red; Synonymous mutation in blue	*COL2A1* intron 47-exon 48	GGCTCCTGTCCTGGCCCTGACCTGACTCAAT CGGTGTCTGTCTTGTTCCCAGGGTCCTCA ATGCCCGCGAGGTGACAAAGGAGAGGCTG GAGAGCCTGGCGAGAGAGGCCTGAAGGG ACACC	
COL2A1-1113mut – mutation screening PCR	*COL2A1* exon 47–48	ATGGGACCCTCAGGACCAGC/ CCTCTCCTTTGTCACCTCGC	
COL2A1-1113scr – PCR for gDNA sequencing	*COL2A1* exon 47–intron 47	ATGGGACCCTCAGGACCAGC/ AGAAACTGCTTAGGGTGATCC	
Single primer for sequencing reaction	*COL2A1* exon 47	ATGGGACCCTCAGGACCAGC	
COL2A1 cDNA Seq – qPCR and sequencing	*COL2A1* exon 46-exon 50	GGGTGATCGTGGTGAGACTG/ GGTTTCGCCTGATCGTCCAC	
Mesoderm (cartilage) marker- qPCR	*SOX9*	AAGTCGGTGAAGAACGGGC/ TCTCGCTTCAGGTCAGCCTT	
	COL2A1	TCACGTACACTGCCCTGAAG/ GCCCTATGTCCACACCGAAT	
Mesoderm (sclerotome) marker- qPCR	*MEOX1*	ACTCGGCTCCGCAGATATGA/ GAACTTGGAGAGGCTGTGGA	
	FOXC2	TGGTATCTCAACCACAGCGG/ CCCGGGACACGTCAGTATTT	
Neuroectoderm marker- qPCR	*PAX6*	TTGCTTGGGAAATCCGAG/ TGCCCGTTCAACATCCTT	
Endoderm marker- qPCR	*SOX17*	GCATGACTCCGGTGTGAATCT/ TCACACGTCAGGATAGTTGCAG	
Housekeeping gene - qPCR	*ACTB*	AAGTCCCTTGCCATCCTAAAA/ ATGCTATCACCTCCCCTGTG	

4.2. CRISPR/Cas9-mediated gene editing

sgRNA targeting COL2A1 was designed using a CRISPR design tool (http://crispr.mit.edu/). sgRNA oligonucleotides were annealed and ligated into pSMART-sgRNA (Sp) plasmid (Addgene # 80427) then sequenced to confirm that no errors had been introduced. Control MCRIi019-A iPSCs were harvested with TrypLE (Thermo Fisher Scientific) and resuspended in Buffer R at a final concentration of 1×10^7 cells/ml. For electroporation, 100 µl of the cell suspension was added to a tube containing 5 ug of in vitro transcribed Cas9geminin mRNA, 2 µg pSMART-COL2A1-sgRNA plasmid and 10 µM of the oligodeoxynucleotide (ODN) repair template incorporating the mutation and synonymous

base changes (Fig. 1B) for PCR screening (Integrated DNA Technologies) (Howden et al., 2018). Cells were electroporated using the Neon® Transfection kit (Thermo Fisher Scientific) at 1100 V, 30 ms, 1 pulse. Electroporated cells were 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 the E8 medium.

4.3. PCR for screening and sequencing

gDNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Clones were screened using allele-specific PCR primers that targeted COL2A1 p.G1113C mutation. PCR used GoTaq Green Mastermix (Promega) with primer sets specified in 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. PCR products were analysed by agarose gel electrophoresis. Clones positive for wild-type and mutant allele specific PCRs were further screened by Sanger sequencing using the BigDye™ Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific). To confirm the mutation at the mRNA level, 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. qPCR primers are listed in Table 2.

4.4. Flow cytometry analysis

Cells were dissociated using TrypLE and incubated with conjugated antibodies to cell surface proteins TRA-1–60 and SSEA4 (Table 2) diluted in PBS containing 2% fetal bovine serum 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 an LSR II (BD Biosciences) and FCS Express software.

4.5. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 10 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 h at room temperature. Nuclei were stained with DAPI (1 μg/ml) and cells visualized by Observer Z.1 microscope (Zeiss). Antibodies and their working dilutions are listed in Table 2.

4.6. 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 qPCR for lineage-specific markers.

4.7. Molecular karyotyping and SNP analysis

Genomic DNA was isolated from MCRIi019-A-7 cells at passage 24 and analyzed (Victorian Clinical Genetics Service, Murdoch Children's Research Institute, Australia) using an Infinium CoreExome-24 v1.1 SNP array (Illumina). MCRIi0019-A-7 was compared to the parental line, MCRIi019-A using SNPduo (http://pevsnerlab.kennedykrieger.org/SNPduo/).

4.8. Mycoplasma detection

iPSCs 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.

Data availability

No data was used for the research described in the article.

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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.2021.102515.

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