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Lab Resource: Multiple Cell Lines

CRISPR/Cas9 gene editing of a SOX9 reporter human iPSC line to produce two *TRPV4* patient heterozygous missense mutant iPSC lines, MCRIi001-A-3 (*TRPV4* p.F273L) and MCRIi001-A-4 (*TRPV4* p.P799L)

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A B S T R A C T

To produce *in vitro* models of human chondrodysplasias caused by dominant missense mutations in *TRPV4,* we used CRISPR/Cas9 gene editing to introduce two heterozygous patient mutations (p.F273L and p.P799L) into an established control human iPSC line. This control line expressed a fluorescent reporter (tdTomato) at the *SOX9* locus to allow real-time monitoring of cartilage differentiation by SOX9 expression. Both *TRPV4* mutant iPSC lines had normal karyotypes, expressed pluripotency markers, and could differentiate into cells representative of the three embryonic germ layers. These iPSC lines, with the parental isogenic control, will be used to study *TRPV4* chondrodysplasia mechanisms and explore therapeutic approaches.

1. Resource Table

Unique stem cell lines identifier	MCRIi001-A-3
	MCRIi001-A-4
Alternative names of stem cell lines	SOX9tdTom-*TRPV4* p.F273L (MCRIi001-A-3)
	SOX9tdTom *TRPV4* p.P799L (MCRIi001-A-4)
Institution	Murdoch Children's Research Institute, Melbourne, Australia
Contact information of distributor	Associate Professor Shireen Lamandé shireen.lamande@mcri.edu.au
Type of cell lines	iPSC
Origin	Human
Cell Source	Peripheral blood mononuclear cell-derived human iPSC line MCRIi001-A-2
Clonality	Clonal
Method of reprogramming	Transgene free Sendia Virus
Multiline rationale	Generate two *TRPV4* human chondrodysplasia patient iPSC for disease modelling
Gene modification	Yes
Type of modification	Induced mutation
Associated disease	Familial digital arthropathy brachydactyly (OMIM #606835; TRPV4 p.F273L); Metatropic dysplasia (OMIM#156530; *TRPV4* p.P799L)
Gene/locus	*TRPV4* c.819C > G p.F273L
	TRPV4 c.2396C > T p.P799L
Method of modification	CRISPR/Cas9
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	March 2018
Cell line repository/bank	https://hpscreg.eu/cell-line/MCRIi001-A-3
	https://hpscreg.eu/cell-line/MCRIi001-A-4
Ethical approval	RCH Human Research Ethics Committee 35121A; MCRI Animal Ethics Committee A788

2. Resource utility

These two *TRPV4* mutant iPSC lines, together with the parental isogenic control iPSC line, provide *in vitro* experimental models to study the molecular mechanisms of how these human patient disease mutations affect TRPV4 channel function and cause cartilage pathology in these chondrodysplasias Table 1.

3. Resource details

TRPV4 (Transient receptor potential cation channel subfamily V

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Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
MCRIi001-A-3	*TRPV4* p.F273L	Male	60	Caucasian	*TRPV4 c.819C > G*	Familial Brachydactyly Arthropathy OMIM #606835
MCRIi001-A-4	*TRPV4* p.P799L	Male	60	Caucasian	*TRPV4 c.2396C > T*	Metatropic dysplasia OMIM 156,530

member 4) is a calcium permeable non-selective cation channel expressed in many tissues and involved in a range of physiological functions, including acting as mechanosensors and activated by hypotonic cell swelling. Channelopathy mutations in *TRPV4* can cause skeletal dysplasias as well as a range of neurological motor function disorders. In this study we used CRISPR/Cas9 gene editing to generate two human iPSC lines with *TRPV4* skeletal dysplasia mutations (*TRPV4* c.819C > G; p.F273L) causing familial digital arthropathy brachydactyly, an inherited arthropathy (FDAB; OMIM #606835) (Lamande et al., 2011) and *TRPV4* c.2396C > T; p.P799L causing metatropic dysplasia (MD; OMIM 156530) (Andreucci et al., 2011), a severe cartilage and bone disorder. These two mutant iPSC cell lines (FDAB; MCRIi001-A-3 and MD; MCRIi001-A-4) and the parental isogenic control line (MCRIi001-A-2) will be used to produce *in vitro* cartilage disease models to explore disease mechanisms and therapies. 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 the appropriate sgRNA and repair templates spanning *TRPV4* exons 5 (FDAB; Fig. 1A) or exon 15 (MD; Fig. 1B). In addition to the patient-specific *TRPV4* mutations, repair templates contained synonymous base changes (Fig. 1A, B, 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 3). Sequencing confirmed the heterozygous *TRPV4* mutations in the genomic DNA and the mRNA in clone MCRIi001-A-3 (c.819C > G; p.F273L; Fig. 1A) and MCRIi001-C-4 (c.2396C > T; p.P799L; Fig. 1B). In addition to the heterozygous *TRPV4* dominant-acting mutations, the synonymous changes were both heterozygous and homozygous (MCRIi001-A-3, Fig. 1A) or homozygous (MCRIi001-A-4, Fig. 1B) indicating that both alleles were targeted during gene-editing recombination events at some of these sites. The location of the mutations in the TRPV4 protein are shown in Fig. 1D.

Both MCRIi001-A-3 and MCRIi001-A-4 had normal stem cell morphology, shown by formation of colonies with well-defined boundaries (Fig. 1C and E). Immunofluorescent staining confirmed the expression of pluripotency marker genes, OCT4 and NANOG in MCRIi001-A-3 (Fig. 1C) and MCRIi001-A-4 (Fig. 1E). Flow cytometry showed that both lines strongly express pluripotency markers, CD9, EPCAM, SSEA-4 and TRA-1-81 (Fig. 1F and G; Table 2). The ability of MCRIi001-A-3 and MCRIi001-A-4 to differentiate into the three embryonic germ layers was determined in teratomas formed by injecting the iPSCs into immunodeficient mice. Staining with haematoxylin and eosin (H&E) demonstrated structures from 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. 1H and I). Mesoderm was represented by cartilaginous tissues (Fig. 1H and I) expressing collagen II (COL2A1) and ectoderm by microtubule associated protein 2, MAP2, staining of structures consistent with peripheral nerve with ganglion cells (Fig. 1H and I). 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 both lines had > 99.9% identity of with the parental line MCRIi001-A (Table 2, Supplementary Fig. 1). Both MCRi001-A-3 and MCRIi001-A-4 were free from mycoplasma contamination (Table 2, Supplementary Fig. 2).

4. Materials and methods

4.1. Cell culture

The human iPSCs were cultured at 37 °C, 5% CO_2 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).

4.2. CRISPR/Cas9-mediated gene editing

The sgRNAs to target *TRPV4* were designed using a CRISPR design tool (http://benchling.com/molecular-biology). The sgRNA oligonucleotides were annealed and ligated into pSpCas9(BB)-2A-GFP (a gift from Feng Zhang; Addgene plasmid #48138; http://n2t.net/addgene:48138; RRID:Addgene_48138). The repair templates were single stranded donor oligonucleotides (ssODN) containing the *TRPV4* mutations (c.819C > G; p.F273L; 228 nt and c.2396C > T; p.P799L; 229 nt) and synonymous changes (Integrated DNA Technologies). For electroporation, 1×10^6 cells (MCRIi001-A-2) were transfected with 2 μg sgRNA-pSpCas9-GFP plasmid and 10 μM ssODN repair template 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.

4.3. PCR for screening and sequencing

Genomic DNA was extracted using a DNAeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. Mutant allele specific PCRs were used to identify gene-edited iPSCs using GoTaq Hot Start polymerase (Promega) (for primers see Table 3). 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 mutant allele specific PCRs were further screened for correct gene-editing by PCR and sequencing using the BigDye™ Terminator v3.1 cycle sequencing kit (Thermo Fisher). iPSCs were differentiated into sclerotome (Loh et al., 2016), RNA extracted using Trizol (Invitrogen) and 500 ng 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.

4.4. Flow cytometry analysis

Cells were dissociated using TrypLE (Thermo Fisher) and 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 3). Propidium iodide (Sigma) was used to identify dead cells.

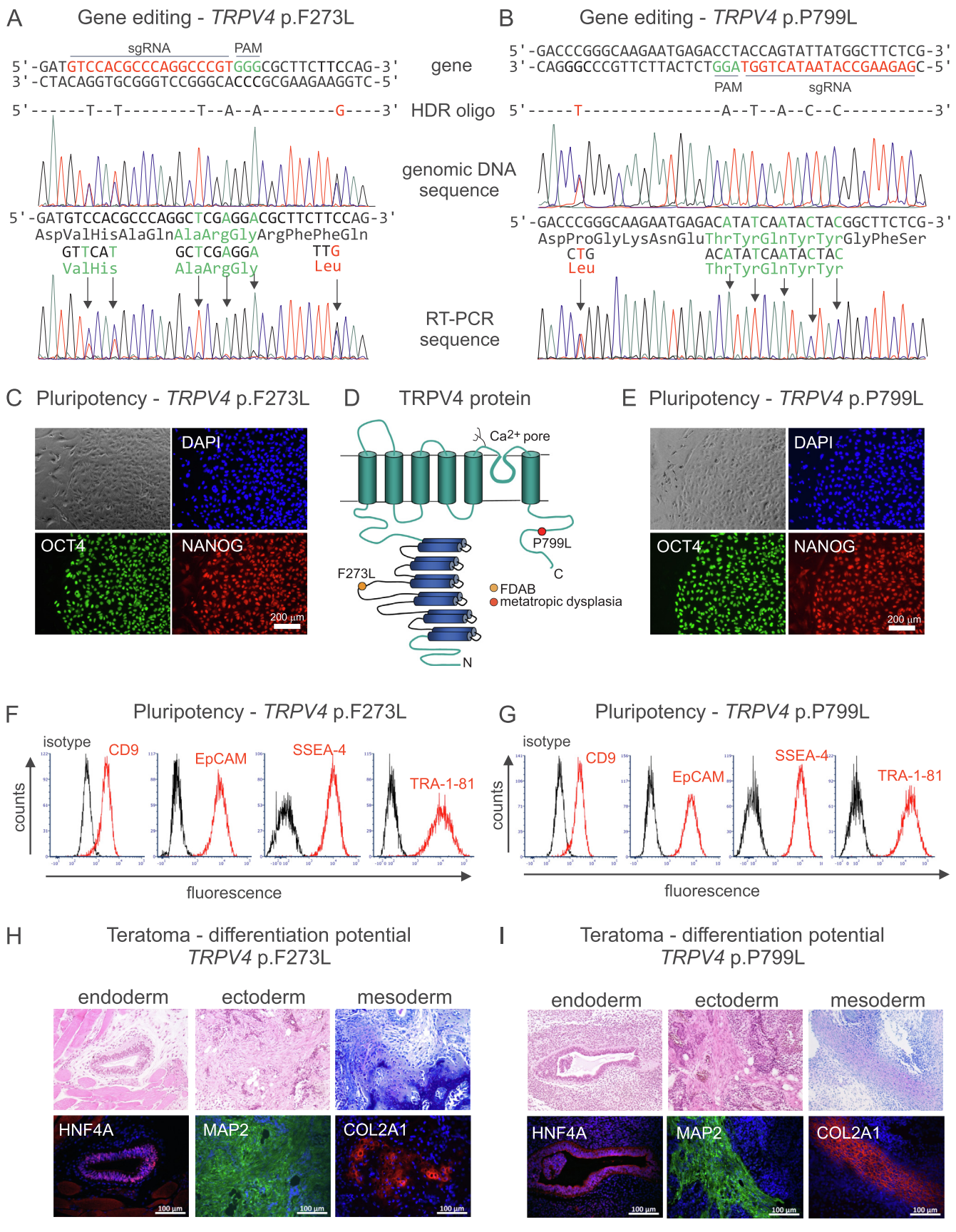

Fig. 1. Ellular and molecular characterization.

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	MCRIi001-A-3 Normal MCRIi001-A-4 Normal	Fig. 1, panel C Fig. 1, panel E
Phenotype	Qualitative analysis	OCT4 and NANOG MCRIi001-A-3 Positive MCRIi001-A-4 Positive	Fig. 1, panel C Fig. 1, panel D
	Quantitative analysis	MCRIi001-A-3; CD9 (95.07%), EpCAM (100%); SSEA-4 (99.97%); TRA-1–81 (99.53%) MCRIi001-A-4; CD9 (95.11%), EpCAM (99.99%); SSEA-4 (99.94%); TRA-1–81 (99.91%)	Fig. 1, panel F Fig. 1, panel G
Genotype	SNP array (resolution 0.5 Mb)	MCRIi001-A-3 46XY No aneuploidies detected MCRIi001-A-4 46XY No aneuploidies detected	Supplementary Fig. 1
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	Supplementary Fig. 1
Mutation analysis	Sequencing	MCRIi001-A-3 Heterozygous *TRPV4* p.F273L gene edited allele confirmed at genomic DNA and mRNA expression level MCRIi001-A-4 Heterozygous *TRPV4* p.F273L gene edited allele confirmed at genomic DNA and mRNA expression level	Fig. 1, panel A Fig. 1, panel B
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR. Both lines negative	Supplementary Fig. 2
Differentiation potential	Teratoma formation (Immunofluorescence)	Both lines positive for germ layer markers. Endoderm: HNF4A Mesoderm: COL2A1 Ectoderm: MAP2	Fig. 1, panel H,I
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers (flow cytometry)	APC anti-human TRA-1–81	1:100	R and D systems Cat# FAB8495A, AB_2687608
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
Pluripotency marker (immunohistochemistry)	Anti-Human Nanog Monoclonal Antibody, clone hNanog.1	1/200	Thermo Fisher Cat# 14–5769-80, RRID: AB_467573
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 (flow cytometry)	BV 421 Mouse IgG2bκ	1/100	BioLegend Cat# 400157, RRID: AB_10897939
Isotype control (flow cytometry)	FITC Mouse IgG1κ	1/100	BD Biosciences Cat# 555748, RRID: AB_396090
Isotype control (flow cytometry)	APC Mouse IgG1 κ Isotype Control	1:100	BioLegend Cat# 555751, AB_398613
Isotype control (flow cytometry)	Alexa Fluor 647 Mouse IgM, κ	1:100	BioLegend Cat# 401618; RRID: AB_2802167

Primers

	Target	**Forward/Reverse primer (5′-3′)**
sgRNA F273L mutant	*TRPV4* exon 5	GTCCACGCCCAGGCCCGT/ ACGGGCCTGGGCGTGGAC
sgRNA P799L mutant	*TRPV4* exon 15	GAGAAGCCATAATACTGGT/ ACCAGTATTATGGCTTCTC
Targeted F273L mutant allele PCR screening	*TRPV4* intron 4 and exon 5 (271 bp)	ATCCCAGGGAATATCCAAGGAC/ GTCCTCGAGCCTGGGCATGA
F273L genomic PCR and sequencing	*TRPV4* intron 4 and intron 5 (768 bp)	ATCCCAGGGAATATCCAAGGAC/TTGAACTCTTGACCTCAGGTGA
F273L RT-PCR and sequencing	*TRPV4* c.585–1700 (1116 bp)	GCCCAAGGCCTTGCTGAACC/ TAGAGGGCTGCTGAGACGATCACC
Targeted P799L mutant allele PCR screening	*TRPV4* intron 14 and exon 15 (286 bp)	TCCTGACCTCAAATGACCCACC/ATGCGAGAAGCCGTAGTATTGA
P799L genomic PCR and sequencing	*TRPV4* intron 14 and intron 15 (357 bp)	CCAGCCTTGTTATGTATATGTTAC/ CGTGTCTACAGAATGAGAGAGTGTG
P799L RT-PCR and sequencing	*TRPV4* c.1541–2617 (1077 bp)	AGGTCATTACGCTCTTCACTGGGG/ CCTAGAGCGGGGCGTCATCAGTC

Samples were analyzed using a BD LSRII (BD Biosciences) using BD FACSDiva and FCS Express software.

4.5. 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 3.

4.6. 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.

4.7. SNP analysis

Genomic DNA was isolated at passage 5 after gene-editing from MCRIi001-A-3 and MCRIi001-A-4 and analyzed (Victorian Clinical Genetics Service, Murdoch Children's Research Institute, Australia) using an Infinium CoreExome-24 v1.1 SNP array (Illumina). MCRIi001-A-3 and MCRIi001-A-4 were compared to the parental line, MCRIi001-A using SNPduo (http://pevsnerlab.kennedykrieger.org/SNPduo/).

4.8. Mycoplasma detection

hiPSCs were tested for mycoplasma using PCR by Cerberus Sciences (Adelaide, Australia).

Declaration of Competing Interest

The authors declare no conflicts of interest.

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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.2020.101942.

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