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Lab resource: Stem Cell Line

Generation of a SOX9-tdTomato reporter human iPSC line, MCRIi001-A-2, using CRISPR/Cas9 editing

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A B S T R A C T

To develop an iPSC SOX9 reporter line for monitoring differentiation into SOX9 expressing cells such as chondrocytes, cranial neural crest and Sertoli cells, we used gene editing to introduce sequences encoding the tdTomato fluorescent protein into the SOX9 locus. The gene-edited line had a normal karyotype, expressed pluripotency markers and differentiated into cells representative of the three embryonic germ layers. Endogenous SOX9 expression was undisturbed and the tdTomato fluorescent reporter mirrored SOX9 mRNA expression. This iPSC line will be useful for assessing iPSC differentiation into SOX9-expressing cells and enrichment by cell sorting.

Resource table

Unique stem cell line identifier	MCRIi001-A-2
Alternative name(s) of stem cell line	MCRIi001-A-SOX9tdTom; PB001-SOX9tdTom
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	Introduction of tdTomato fluorescent protein reporter sequence at the SOX9 locus
Associated disease	N/A
Gene/locus	SOX9
Method of modification	CRISPR/Cas9
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	January 2019
Cell line repository/bank	https://hpscreg.eu/cell-line/MCRIi001-A-2
Ethical approval	RCH Human Research Ethics Committee 35121A; MCRI Animal Ethics Committee A788

Resource utility

This fluorescent SOX9-reporter human iPSC line will be a useful tool for prospective cell sorting and analysis and monitoring the *in vitro* differentiation of SOX9-expressing cells during developmental processes related to skeletal formation and gonad development.

Resource Details

The transcription factor SOX9 plays a central role in orchestrating differentiation of several cell lineages. While chondrocyte differentiation is a key SOX9 regulated process, other developmentally important processes include cranial neural crest and Sertoli cell differentiation (Symon and Harley, 2017). We used Cas9 gene editing to introduce sequences encoding the tdTomato fluorescent protein with an upstream T2A into the SOX9 locus (Fig 1A) of a control human iPSC line, MCRIi001-A (https://hpscreg.eu/cell-line/MCRIi001-A). The T2A peptide sequence was incorporated to allow tdTomato reporter protein expression contemporaneously with SOX9, but without the possible functional effects of a SOX9-TdTomato fusion protein. MCRIi001-A

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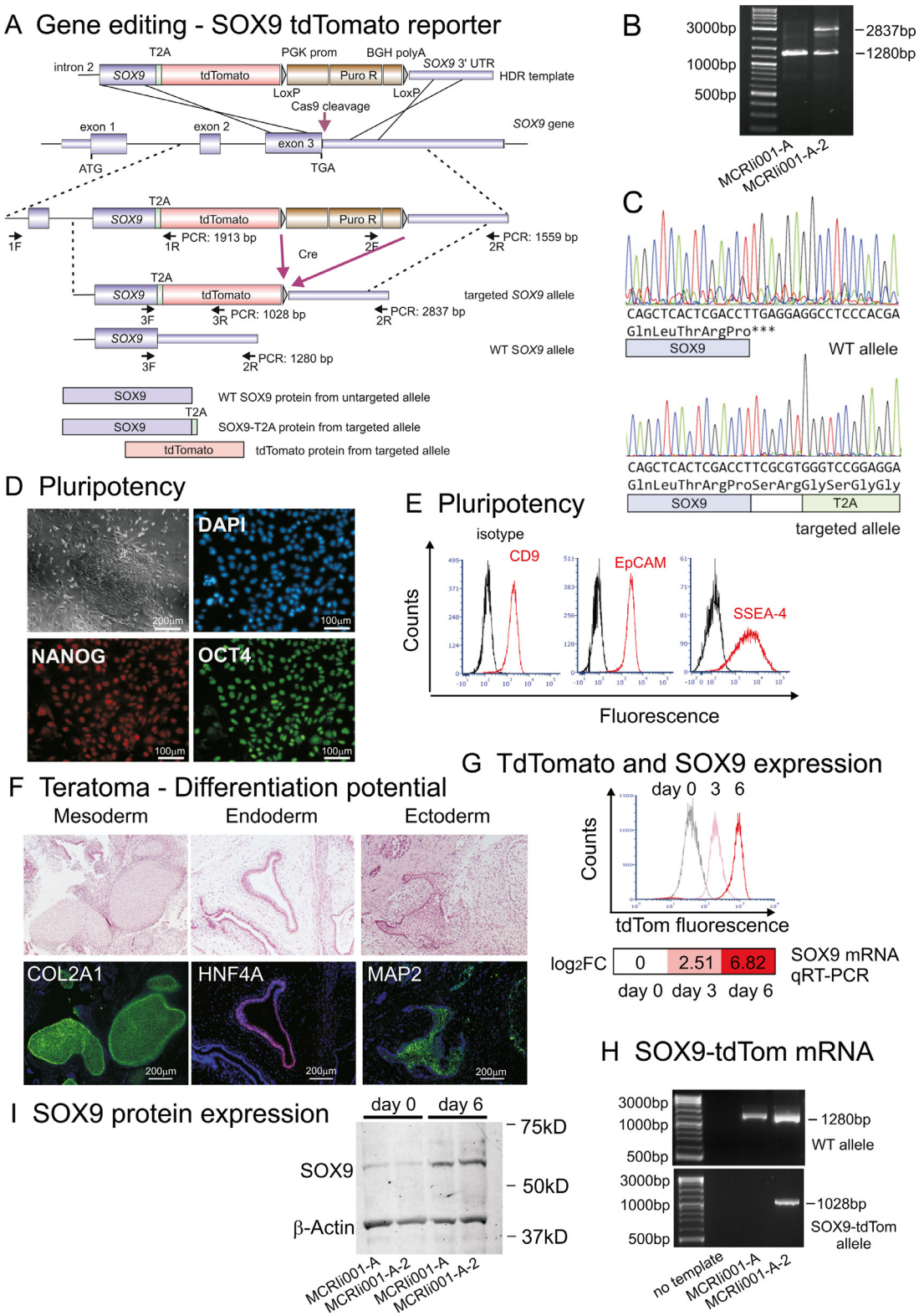

Fig. 1. .

iPSCs were transfected with an homology directed repair (HDR) template that was targeted to the SOX9 stop codon in exon 3 and encoded the T2A, tdTomato and floxed puromycin resistance cassette (Fig. 1A). Transfected clones were selected with puromycin and a correctly targeted heterozygous clone was identified using PCR primer sets 1F + 1R and 2F + 2R, which generated amplicons of 1913 bp and 1559 bp, respectively (Fig. 1A). The targeted clone was subsequently treated with Cre-recombinase to remove the puromycin resistance gene thus generating the SOX9-T2A-tdTom allele (Fig 1A). Clones were screened by PCR using primers flanking the T2A-tdTomato sequence (Fig 1A, primers 3F + 2R), which demonstrated the presence of the WT allele (1280 bp) and the SOX9-T2A-tdTomato-containing allele (2837 bp) in a heterozygous iPSC clone, MCRIi001-A-2 (Fig 1B). Sequencing the mutant and WT alleles confirmed the correct in-frame insertion of the

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel D
Phenotype	Qualitative analysis (Immunofluorescence)	OCT4 and NANOG Positive	Fig. 1 panel D
	Quantitative analysis (Flow cytometry)	CD9: 97.87% EpCAM: 98.5% SSEA-4: 93.18%	Fig. 1 panel E
Genotype	SNP array (resolution 0.5 Mb)	46XY No aneuploidies were 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	Heterozygous gene edited SOX9-tdTomato reporter allele confirmed.	Fig. 1 panel B (PCR) Fig. 1 panel C (sequencing)
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR. Negative	Supplementary Fig. 2
Differentiation potential	Teratoma formation	Endoderm: HNF4A Ectoderm: MAP2 Mesoderm: COL2A1	Fig. 1 panel F

Table 2
Reagents 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# 324,220, RRID: AB_2,563,847
Pluripotency marker (flow cytometry)	Mouse anti-CD9 Monoclonal Antibody, FITC conjugated, Clone M-L13	1/20	BD Biosciences Cat# 555,371, RRID: AB_395,773
Pluripotency marker (flow cytometry)	Alexa Fluor 647 anti-human SSEA-4 antibody	1:40	BioLegend Cat# 330,408, RRID: AB_1,089,200
Pluripotency marker (immunohistochemistry)	Anti-Human Nanog Monoclonal Antibody, clone hNanog.1	1/200	ThermoFisher Scientific Cat# 14–5769–80, RRID: AB_467,573
Pluripotency marker (immunohistochemistry)	Oct-4A (C30A3) Rabbit mAb	1/400	Cell Signaling Technology Cat# 2840, RRID: AB_2,167,691
Differentiation marker (immunohistochemistry)	Anti-Collagen Type II Antibody, clone 6B3 (COL2A1)	1/150	Merck Cat# MAB8887, RRID: AB_2,260,779
Differentiation marker (immunohistochemistry)	HNF4A Monoclonal Antibody (K9218)	1/300	ThermoFisher Scientific Cat# MA1-199, RRID: AB_2,633,309
Differentiation marker (immunohistochemistry)	Monoclonal Anti-MAP2 (2a + 2b) antibody	1/250	Merck Cat# M1406, RRID: AB_477,171
Differentiation marker (immunoblotting)	Anti-Sox9 Antibody	1/2000	Millipore Cat# AB5535, RRID: AB_2,239,761
Secondary antibody	Donkey anti-Mouse IgG (H&L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	1/1000	ThermoFisher Scientific Cat# A21203, RRID: AB_2,535,789
Secondary antibody	Goat anti-Rabbit IgG (H&L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1/1000	ThermoFisher Scientific Cat# A11008, RRID: AB_143,165
Secondary antibody	Goat anti-Mouse IgG (H&L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1/500	ThermoFisher Scientific Cat# A11029, RRID: AB_2,534,088
Isotype control	BV 421 Mouse IgG2bκ	1/100	BioLegend Cat# 400,341, RRID: AB_10,898,160
Isotype control	FITC Mouse IgG1κ	1/100	BD Biosciences Cat# 555,748, RRID: AB_396,090
Isotype control	Alexa Fluor 647 Mouse IgM, κ	1/100	BioLegend Cat# 401,618; RRID: AB_2,802,167
Primers			
	Target	Forward/Reverse primer (5′–3′)	
SOX9-exon3-sgRNA	*SOX9* exon 3	CACCGCCATCTTCGCCCTTCGT/AAACACGAAGGGCGAAGATGGC	
Infusion cloning SOX9 5′ homology arm	*SOX9* intron 2-exon 3, 1000 bp	TATCGAATTTACGCGCATTGGGCGACTTATCTCCGGT/ CTCCGGACCCACGCGAAGGTCGAGTGAGCTGTGTGTAG	
Infusion cloning SOX9 3′ homology arm	*SOX9* exon 3, 1000 bp	AAGTTATCCGGCGCGGGGCGAAGATGGCCGAGATGAT/ AGCCTCGATGGCGCGCAGTGTGCTCGGGCACTTATTGG	
Homologous recombination 5′-end (1F + 1R)	*SOX9* intron 1-tdTomato, 1913 bp	GCAGGAGGGAAGATGGAGTTGT/ACTCTTTGATGACCTCCTCGCC	
Homologous recombination 3′-end (2F + 2R)	PuroR-SOX9 exon 3, 1559 bp	CAACCTCCCCTTCTACGAGCG/TTCTGAGAGGCACAGGTGACAG	
PuroR excision by Cre recombinase and presence of WT allele (3F + 2R)	*SOX9* exon 3, 2837 bp for SOX9-T2A-tdTom allele, 1280 bp for WT allele	CCACCAGAACTCCAGCTCCTAC/TTCTGAGAGGCACAGGTGACAG	
RT-PCR (3F + 3R)	*SOX9* exon 3 and tdTom, 1208 bp for SOX9-T2A-tdTom mRNA	CCACCAGAACTCCAGCTCCTAC/ACTCTTTGATGACGGCCATGTTGTTG	
quantitative RT-PCR	*SOX9*	AAGTCGGTGAAGAACGGGC/TCTCGCTTCAGGTCAGCCTT	
quantitative RT-PCR	*ACTB*	AAGTCCCTTGCCATCCTAAAA/ATGCTATCACCTCCCCTGTG	

reporter cassette and an intact WT allele (Fig 1C). Genome SNP array confirmed no aneuploidies or large deletions or insertions and greater than 99.9% identity of MCRIi001-A-2 with the parental line (Table 2, Supplementary Fig. 1). It should be noted that the SNP analysis does not preclude the presence of balanced translocations. MCRi001-A-2 was free from mycoplasma (Table 2, Supplementary Fig. 2). MCRIi001-A-2 colonies had normal stem cell morphology with discrete and well-defined boundaries and a high nucleus to cytoplasm ratio (Fig. 1D). Immunofluorescent staining confirmed expression of nuclear pluripotency markers, OCT4 and NANOG (Fig. 1D) and flow cytometry showed the

cell population co-expressed pluripotency markers SSEA-4, CD9 and EpCAM (Fig. 1E). This iPSC line differentiated into the three embryonic germ layers in teratomas produced in immunodeficient mice. Histology revealed structures consistent with cartilage (mesoderm), intestinal epithelial-like tissue (endoderm) and squamous epithelium (ectoderm). The identity of these was confirmed by immunostaining for mesoderm (collagen II, COL2A1), endoderm (hepatocyte nuclear factor 4 alpha, HNF4A) and ectoderm (microtubule associated protein 2, MAP2) (Fig 1F). *In vitro* differentiation to sclerotome (Loh et al., 2016) was used to initiate SOX9 expression. SOX9 mRNA was upregulated at day 3 of sclerotome differentiation and further upregulated at day 6, and this increase was mirrored by increased tdTomato fluorescence detected by flow cytometry (Fig 1G). RT-PCR showed that MCRIi001-A-2 expressed WT SOX9 mRNA and SOX9-tdTOM mRNA (Fig 1H). Western blotting confirmed that SOX9 protein expression was upregulated during sclerotome differentiation and that expression in MCRIi001-A-2 was similar to the parental line, MCRIi001-A (Fig 1I). This indicated that introduction of the T2A-tdTomato cassette at the SOX9 3′-end did not disrupt SOX9 protein expression.

1. Materials and methods

1.1. Cell culture

The 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 passaged (1:6 split) every 3 days with 0.5 mM EDTA in PBS.

1.2. Cas9-mediated gene editing

The sgRNA oligonucleotides were annealed and ligated into pSpCas9(BB) − 2A-GFP (PX458) (Addgene # 48,138). To generate the HDR template, 1000 bp of the SOX9 3′-untranslated region was PCR amplified (AccuPrime Taq DNA polymerase, Thermo Fisher Scientific) and cloned (In-Fusion HD Cloning Kit, Clontech) downstream of the puromycin resistance cassette into p2A-tdTom-PuroR, a vector containing T2A-tdTomato-LoxP-PGK promoter-PuroR-BGH polyA-LoxP'. Next, 1000 bp of the SOX9 gene upstream of the stop codon was PCR amplified (CloneAmp HiFi PCR Premix, Clontech) and cloned upstream of the T2A sequence. The PCR primers (Table 2) were designed to replace the SOX9 stop codon with TCGCGT (SerArg) and be in-frame with the T2A sequence (Fig 1C). Both SOX9 homology arms were sequenced to ensure that errors had not been introduced. 1×10^6 cells were transfected with 2 μg sgRNA/Cas9/GFP plasmid and 5 μg of the HDR template using the Neon® Transfection kit (Thermo Fisher Scientific) (Howden et al., 2016). 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 1 μg/ml puromycin (Gibco) was added to select for gene edited cells. Puromycin resistant clones were expanded and a fraction of each colony collected for PCR screening and sequencing. To excise the puromycin resistance gene, ~1×10^6 cells of a successfully targeted heterozygous clone were transfected with 3 μg of the Cre recombinase-expressing plasmid, pCre-IRES-PuroR (a gift from Darrell Kotton, Addgene plasmid # 30,205), and 0.2 μg pEF-BOS-GFP as above. On the third day, GFP-positive single cells were flow sorted (FACSAria Fusion Cell Sorter, BD Biosciences) into 96 well plates plated with MEFs. Single cell clones were expanded and screened by PCR and sequencing.

1.3. PCR for screening and sequencing

PCRs used GoTaq G2 Hot Start polymerase (Promega) (for primers see Table 2) and touchdown conditions; 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 sequenced using the BigDye™ Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific).

1.4. Flow cytometry analysis

Cells were dissociated with TrypLE and incubated with 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. Dead cells were identified using propidium iodide (Sigma). Samples were analyzed using a BD LSRII (BD Biosciences) and BD FACSDiva and FCS Express software.

1.5. Sclerotome differentiation

iPSCs were differentiated into sclerotome (Loh et al., 2016) and analyzed by flow cytometry.

1.6. Quantitative RT-PCR

RNA was extracted from cells using TRIzol (Invitrogen). 1 μg of RNA was used for cDNA synthesis (20μl reaction) using a QuantiTect® Reverse Transcription Kit (Qiagen). qRT-PCR used Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies) in 10 μl reactions in a 384-well plate containing 5 μl 2× SYBR Green Master Mix, 1 μl of 1:10 diluted cDNA and 4 μl of 1 μM primer stock (forward and reverse primers) and a LightCycler® 480 II (Roche).

1.7. SNP analysis

MCRIi001-A cells at passage 19 were used to generate the reporter line and SNP analysis on MCRIi001-A-2 was performed 13 passages later, after CRISPR mediated recombination. Genomic DNA was isolated and analyzed (Victorian Clinical Genetics Service, Murdoch Children's Research Institute, Australia) using an Infinium CoreExome-24 v1.1 SNP array (Illumina). MCRIi001-A-2 was compared to the parental line, MCRIi001-A, using SNPduo analysis (http://pevsnerlab.kennedykrieger.org/SNPduo/).

1.8. Mycoplasma detection

iPSCs were tested for Mycoplasma by Cerberus Sciences (Adelaide, Australia).

1.9. 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, blocked in PBST (PBS + 0.1% Tween-20) containing 3% bovine serum albumin (BSA) for 30 min and stained overnight at 4 °C with primary antibodies diluted in PBST containing 1% BSA. Secondary antibodies were applied for 1 hr at room temperature. Antibodies are listed in Table 2. Nuclei were stained with DAPI (1 μg/ml) and cells visualized by fluorescent microscopy (Olympus IX70).

1.10. Teratoma production and analysis

Teratomas were generated in immunodeficient mice as described (International Stem Cell, 2018). Teratoma tissue was fixed in CONFIX (Australian Biostain) and paraffin-embedded. Sections (5 μm) were stained with haematoxylin eosin or specific antibodies as above.

1.11. Western blotting

Proteins were extracted, separated by gel electrophoresis, transferred to membranes and detected as described (Gandolfi et al., 2016).

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2019.101689.

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