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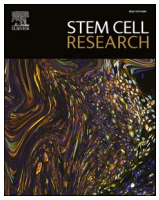

Generation of a human ACTA1-tdTomato reporter iPSC line using CRISPR/Cas9 editing

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A B S T R A C T

We used gene editing to introduce DNA sequences encoding the tdTomato fluorescent protein into the α -skeletal actin 1 (ACTA1) locus to develop an ACTA1-tdTomato induced pluripotent stem cell reporter line for monitoring differentiation of skeletal muscle. This cell line will be used to better understand skeletal muscle maturation and development *in vitro* as well as provide a useful tool for drug screening and the evaluation of novel therapeutics for the treatment of skeletal muscle disease.

1. Resource Table

Unique stem cell line identifier	MCRIi010-A-1
Alternative name(s) of stem cell line	ACTA1tdTom; PB010.5-ACTA1tdTom
Institution	Murdoch Children's Research Institute (MCRI)
Contact information of distributor	Peter.houweling@mcri.edu.au
Type of cell line	iPSC
Origin	Human
Additional origin info required for human ESC or iPSC	Age: 20 Sex: Male Ethnicity: Caucasian
Cell Source	Peripheral blood mononuclear cell (PBMC) derived human iPSC line MCRIi010-A
Clonality	Clonal
Associated disease	N/A
Gene/locus	*ACTA1*
Date archived/stock date	
Cell line repository/bank	https://hpscreg.eu/cell-line/MCRIi010-A-1
Ethical approval	Royal Children's Hospital HREC Reference Number: 35121A

2. Resource utility

This fluorescently labelled ACTA1-tdTomato reporter iPSC line will be a useful to explore the developmental processes relating to skeletal muscle maturation and as a tool to model ACTA1-related congenital muscle diseases.

3. Resource details

α-Skeletal actin 1 (ACTA1) belongs to the highly conserved family of actin proteins, which play a key role in cell motility, structure and integrity. Up to six actin isoforms have been identified, with α-skeletal actin being a major component (~95 %) of adult skeletal muscle (Bertola,Ott et al., 2008). ACTA1 plays a primary role in skeletal muscle contraction. Mutations in *ACTA1* are known to cause a variety of genetic muscle wasting diseases, including nemaline myopathy (NM). Mutations in α-skeletal actin are responsible for ~ 26 % of all diagnosed cases of NM and are the primary cause of the more severe congenital onset myopathies (Sewry,Laitila et al., 2019).

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The iPSC line, MCRIi010-A, was derived from PBMCs of a healthy male using Sendai virus carrying the reprogramming factors POU5F1 (OCT4), SOX2, KLF4 and MYC (https://hpscreg.eu/cell-line/MCRIi010-A) as previously published (Vlahos,Sourris et al., 2019). We used an established gene editing strategy (Nur Patria,Lilianty et al., 2020) to introduce sequences encoding the tdTomato fluorescent protein with an upstream P2A peptide into the ACTA1 locus (Fig. 1A) to generate the tdTomato tagged ACTA1 reporter iPSC line (https://hpscreg.eu/cell-line/MCRIi010-A-1). The P2A peptide sequence was incorporated to allow tdTomato reporter protein expression contemporaneously with ACTA1 (Fig. 1A). All ACTA1 protein produced will carry the C-terminal 2A tag along with the tdTomaoto reporter.

Flow cytometry confirmed that the MCRIi010-A-1 iPSC line strongly express pluripotency markers CD326, SSEA4 and TRA-1–81 (Fig. 1C). A normal stem cell morphology was observed as characterised by compact colonies with well-defined cell boundaries (Fig. 1D). Droplet digital PCR (ddPCR) also confirmed expression of pluripotency marker *OCT4* mRNA in undifferentiated iPSCs, which was reduced following differentiation to a mesoderm cell lineage (Fig. 1E).

Pluripotency of this iPSC line was shown by direct differentiation into the three germ layers. Definitive endoderm differentiation was confirmed by expression of SOX17 (Fig. 1F). Ectoderm differentiation was demonstrated by the presence of NESTIN and PAX6 (Fig. 1G). Mesoderm lineage was shown by increases in *Brachyury* and *CXCR4* mRNA at day 5 of direct differentiation (Fig. 1H). Furthermore, high levels of tdTomato were seen following terminal differentiation of MCRIi010-A-1 to skeletal muscle myotubes (Fig. 1I).

Genome SNP array analysis confirmed a stable karyotype (Supplementary Fig 1) and a SNP Duo analysis confirmed that MCRIi010-A-1 had > 99.9 % identity to the parental line MCRIi010-A, indicating that the two lines are from the same individual (Table 1, Supplementary Fig 2). Furthermore, we confirmed that the ACTA1 gene copy number was normal following the addition of the td-tomato reporter construct by performing the Infinium GDACyto-8 v1.0 assay (Supplementary Fig. 3 and Fig. 4). Both lines (MCRIi010-A and MCRIi010-A-1) were confirmed to be free from mycoplasma contamination (Table 1, Supplementary Fig 1).

4. Materials and methods

4.1. Cell culture

MCRIi010-A iPSCs were cultured at 37 °C with 5 % CO_2 on Corning® Matrigel-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.

4.2. CRISPR/Cas9-mediated gene editing of patient PBMCs

Gene-editing factors were introduced into the MCRIi010-A parent line using the Neon transfection system (1100 V, 30 ms, 1 pulse). Transfected cells were plated into 2 wells of a Matrigel/MEF coated 6-well dish in mTesR medium. Medium (2 ml) was replaced in each well 48 hrs post-transfection and subsequent media changes were performed every day until individual colonies were isolated for screening.

4.3. PCR screening and sequencing

Gene-edited clones were identified by allele-specific PCR using primers that flank the 5′ and 3′ recombination junctions (ACTA1_scF: CCTCCTTCATCGGTGAGCC and P2A_scR:GAAGTTAGTAGCTCCACTTCC; SV40_scF: TCTAGAGCTCGCTGATCAGC and ACTA1_scR:CTGAGAAACAGCTCTCCAGC) (Table 2) of the *ACTA1* gene. Individual iPSC colonies were picked and expanded in E8 medium. A colony that

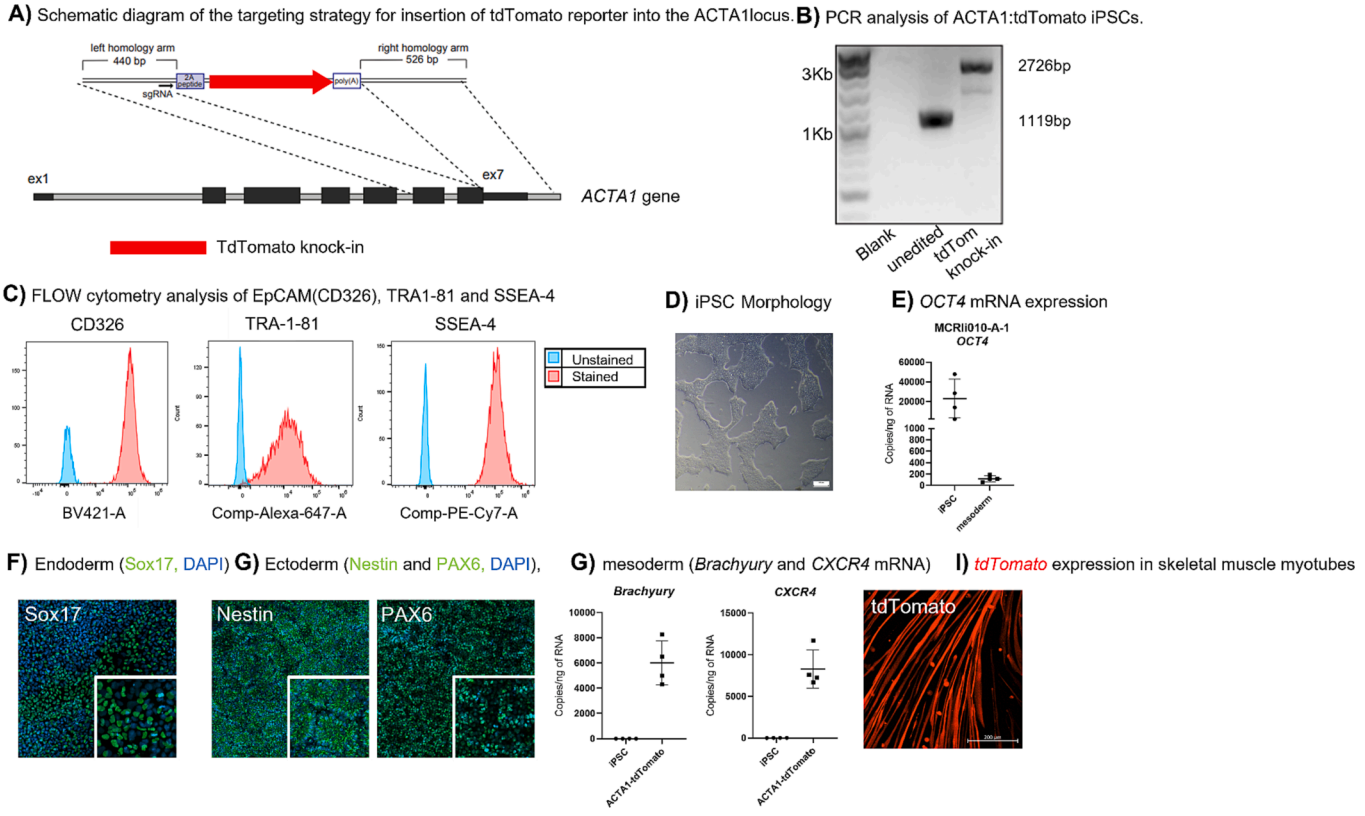

Fig. 1.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	Normal	Fig. 1 panel D
Phenotype	Quantitative mRNA analysis (ddPCR)	*OCT4* mRNA	Fig. 1 panel D
	Quantitative FLOW cytometry analysis	MCRIi010-A-1 CD234: 99. 5 % SSEA: 99.9 % TRA-1–81: 96.0 %	Fig. 1 panel C
Genotype	Infinium GSA-24 v1.0 SNP array (Illumina)	arr(X,Y)x1,(1–22)x2 No aneuploidies detected	Supplementary Fig 1
Identity	SNPDuo comparative analysis of SNParrays	Identical SNP genotypes (>99.9 %) for the entire genome indicating the two samples are from the same individual	Supplementary Fig 2
Mutation analysis (IF APPLICABLE)	N/A		
Microbiology and virology	Mycoplasma	Confirmed negative by PCR	Supplementary Fig 1
Differentiation potential	Directed differentiation	Endoderm: SOX17 Ectoderm: Nestin and PAX6 Mesoderm: Brachyury and CXCR4	Fig. 1, panels E, F, G
List of recommended germ layer markers	**Expression of these markers has to be demonstrated at mRNA (ddPCR) or protein (IF) levels.**	Positive expression for SOX17, Nestin, PAX6, Brachyury and CXCR4 were observed	Fig. 1, panels D-H
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not available	Not available
Genotype additional info (OPTIONAL)	Blood group genotyping	Not available	Not available
	HLA tissue typing	Not available	Not available

contained a high proportion of corrected cells was selected for subcloning to attain a pure population of gene-corrected cells. Subcloning was performed by dissociating cells with TrypLE and plating at low density on MEFs in mTesR. Individual colonies were again isolated and expanded and screened by PCR. A pure subclone which showed homozygous insertion of the tdTomato reporter (as determined using primers ACTA1_scF and ACTA1_scR) was selected for further characterisation.

gDNA was extracted from individual iPSC colonies using a DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's instructions. PCR was performed using GoTaq Green Mastermix (Promega) with primer sets specified in Table 2 and an Applied Biosystems (Veriti) 96-well thermocycler. PCR products were analysed by agarose gel electrophoresis with expected amplicon size for unedited allele = 1119 bp, and tdTomato knock-in allele = 2726 bp (Fig. 1B).

4.4. RNA extraction and digital droplet PCR (ddPCR) quantitation

RNA was extracted using an RNeasy mini kit (Qiagen). Total RNA was quantitated using TapeStation (Agilent Technologies 2200). Diluted RNA concentrations were assessed using Qubit 3.0 fluorometer (Thermo Fisher Scientific). All RNA samples were reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) as per manufacturer guidelines.

ddPCR assays were conducted using 2X QX200 ddPCR EvaGreen Supermix (Biorad) in a twin.tec 96-well plate (Biorad) to a final volume of 24 μl for lipid droplet generation and PCR amplification. Following PCR amplification, the sample plate was loaded on the QX200 Droplet reader (Biorad) and the assay information was entered into the QuantaSoft (BioRad) software.

4.5. Immunocytochemistry

Cells were fixed in 4 % paraformaldehyde for 10 min at room temperature then permeabilized with 0.1 % Triton X-100 in PBS for 10 min at room temperature. Non-specific binding was blocked with 3 % bovine serum albumin in PBS overnight at 4 °C. Cells were incubated with primary antibodies for 2 h at room temperature, followed by secondary antibodies for 1hr (Table 2). Nuclei were stained with DAPI (1 μg/ml) and images were captured with a LSM900 confocal microscope (Leica Microsystems).

4.6. Flow cytometry

Cells were dissociated with TrypLE (Thermo Fisher Scientific) and incubated with conjugated antibodies to cell surface proteins CD326, TRA-1–81 and SSEA4 (Table 2) diluted in PBS containing 2 % foetal bovine serum for 15 min at 4 °C. Cells were then washed with 2 % FBS in PBS, and analysed using a Cytek®Aurora (BD Biosciences), BD FACSDiva and FCS Express software.

4.7. Directed differentiation (endoderm, Neuroectoderm, mesoderm and skeletal muscle myotubes)

iPSCs were differentiated in monolayer culture to either endoderm, ectoderm or mesoderm using the STEMdiff™ Trilineage Differentiation Kit (StemCell Tech). Differentiation was assessed by immunocytochemistry and/or mRNA expression using ddPCR for lineage-specific markers. Terminal differentiation to skeletal muscle myoblasts was achieved using the STEMdiff™ Myogenic Progenitor supplement kit as per manufacturer's instructions. Myoblasts were fused to myotubes using the MyoCult™ Differentiation kit, and imaged using the LSM 900 confocal microscope for tdTomato expression after 5 days of terminal differentiation.

4.8. Molecular karyotyping, SNP analysis and Mycoplasma detection

Cell pellets were provided to the Victorian Clinical Genetics Service (Murdoch Children's Research Institute, Melbourne, Australia) and genomic DNA was analysed using an Infinium GSA-24 v1.0 SNP array and Illumina Infinium GDACyto-8 v1.0 (Illumina) and. Mycoplasma contamination was assessed by PCR (Cerberus Sciences, Adelaide, Australia).

5. Additional files

SNP DUO analysis by the Victorian Clinal Genetics Service (VCGS).

Table 2
Reagents details.

	Antibodies used for immunocytochemistry/flow-cytometry		
	Antibody	**Dilution**	**Company Cat # RRID**
Pluripotency marker	APC anti-human CD326 (EpCAM) antibody	1:50	BioLegend Cat# 324208/9C4, RRID: AB_756081
Endoderm marker	Goat anti-Human SOX17 polyclonal antibody	1:100	R&D Systems Cat# AF1924, RRID: AB_2251134
Ectoderm marker	Mouse anti-nestin antibody, clone 10C2	1:200	Merck Cat# MAB5326, RRID: AB_2251134
Secondary Antibody	Donkey anti-rabbit IgG (H & L) Alexa Fluor 594	1:1000	Thermo Fisher Scientific Cat# A21207, RRID: AB_141637
Secondary Antibody	Goat anti-mouse IgG (H & L) Alexa Fluor 488	1:1000	Thermo Fisher Scientific Cat# A11029, RRID: AB_2534088
Secondary Antibody	Donkey anti-Goat IgG (H & L) Alexa Fluor 488	1:1000	Thermo Fisher Scientific Cat# A32814, RRID: AB_2762838
FACS antibody	Alexa Fluor 647 anti-human SSEA-4 antibody	1:100	BioLegend Cat# 330408, RRID: AB_1089200
FACS antibody	BV421 Mouse Anti-Human TRA-1–81 Antigen	1:20	Becton Dickinson Cat# 562711, RRID: AB_2737738
	Target	**Size of band**	**Forward/Reverse primer (5'-3')**
Pluripotency marker	*OCT4*	261bp	GAAGTGGGTGGAGGAAGCTG/ TAGTCGCTGCTTGATCGCTT
Mesoderm marker	Brachyury	197bp	TGAGACCCAGTTCATAGCGG/ TGCTGGTTCCAGGAAGAAGC
Mesoderm marker	*CXCR4*	155bp	GGACCTGTGGCCAAGTTCTTA/ AAACGTTCCACGGGAATGGA
Knock-in template (ODN) sequence:		2564bp	*tctgctcacgctccccgccgcggtccccagGTATG GAGTCGGCGGGCATTCACGAGACC ACCTACAACAGCATCATGAAGTGTG ACATCGACATCAGGAAGGACCTGTA TGCCAACAACGTCATGTCGGGGGG CACCACGATGTACCCTGGGATCGCT GACCGCATGCAGAAAGAGATCACCG CGCTGGCACCCAGCACCATGAAGAT CAAGgtgggtggtggcctgcgcgggctgtcggcg gggtgggctccagggtgaggtctccccacctcacgc gctgtcttgcagATCATCGCCCCGCCGGA GCGCAAATACTCGGTGTGGATCGGC GGCTCCATCCTGGCCTCGCTGTCCA CCTTCCAGCAGATGTGGATCACCAA GCAGGAGTACGACGAGGCCGGCCC TTCCATCGTCCACCGCAAATGCTTC GGAAGTGGA*GCTACTAACTTCAGT

(*continued on next page*)

Table 2 (*continued*)

			CTTCTGAAGCAGGCTGGTGACGTC GAAGAGAACCCTGGACCTGGTCT TAGACCTATGGTGAGCAAGGGCG AGGAGGTCATCAAAGAGTTCATG CGCTTCAAGGTGCGCATGGAGGG CTCCATGAACGGCCACGAGTTCG AGATCGAGGGCGAGGGCGAGGGC CGCCCCTACGAGGGCACCCAGAC CGCCAAGCTGAAGGTGACCAAGG GCGGCCCCCTGCCCTTCGCCTGGG ACATCCTGTCCCCCCAGTTCATGT ACGGCTCCAAGGCGTACGTGAAG CACCCCGCCGACATCCCCGATTAC AAGAAGCTGTCCTTCCCCGAGGG CTTCAAGTGGGAGCGCGTGATGA ACTTCGAGGACGGCGGTCTGGTG ACCGTGACCCAGGACTCCTCCCTG CAGGACGGCACGCTGATCTACAA GGTGAAGATGCGCGGCACCAACT TCCCCCCCGACGGCCCCGTAATGC AGAAGAAGACCATGGGCTGGGAG GCCTCCACCGAGCGCCTGTACCCC CGCGACGGCGTGCTGAAGGGCGA GATCCACCAGGCCCTGAAGCTGA AGGACGGCGGCCACTACCTGGTG GAGTTCAAGACCATCTACATGGC CAAGAAGCCCGTGCAACTGCCCG GCTACTACTACGTGGACACCAAG CTGGACATCACCTCCCACAACGA GGACTACACCATCGTGGAACAGT ACGAGCGCTCCGAGGGCCGCCAC CACCTGTTCCTGGGGCATGGCACC GGTAGCACCGGCAGCGGCAGCTC CGGTACCGCCTCCTCCGAGGACA ACAACATGGCCGTCATCAAAGAG TTCATGCGCTTCAAGGTGCGCATG GAGGGCTCCATGAACGGCCACGA GTTCGAGATCGAGGGCGAGGGCG AGGGCCGCCCCTACGAGGGCACC CAGACCGCCAAGCTGAAGGTGAC CAAGGGCGGCCCCCTGCCCTTCGC CTGGGACATCCTGTCCCCCCAGTT CATGTACGGCTCCAAGGCGTACG TGAAGCACCCCGCCGACATCCCC GATTACAAGAAGCTGTCCTTCCCC GAGGGCTTCAAGTGGGAGCGCGT

(*continued on next page*)

Table 2 (*continued*)

			GATGAACTTCGAGGACGGCGGTC TGGTGACCGTGACCCAGGACTCCT CCCTGCAGGACGGCACGCTGATC TACAAGGTGAAGATGCGCGGCAC CAACTTCCCCCCCGACGGCCCCGT AATGCAGAAGAAGACCATGGGCT GGGAGGCCTCCACCGAGCGCCTG TACCCCCGCGACGGCGTGCTGAA GGGCGAGATCCACCAGGCCCTGA AGCTGAAGGACGGCGGCCACTAC CTGGTGGAGTTCAAGACCATCTAC ATGGCCAAGAAGCCCGTGCAACT GCCCGGCTACTACTACGTGGACA CCAAGCTGGACATCACCTCCCAC AACGAGGACTACACCATCGTGGA ACAGTACGAGCGCTCCGAGGGCC GCCACCACCTGTTCCTGTACGGCA TGGACGAGCTGTACAAGTAATAG GCGGCCGCTAAGATATCTTTCTAG AGCTCGCTGATCAGCCTCGA AATAAAGCAATAGCATCACAAAT TTCACAAATAAAGCATTTTTTTCA CTGC*TTGAAACACACTCCACCTCCA* *GCACGCGACTTCTCAGGACGACGAA* *TCTTCTCAATGGGGGGGCGGCTGA* *GCTCCAGCCACCCCGCAGTCACTTT* *CTTTGTAACAACTTCCGTTGCTGCCA* *TCGTAAACTGACACAGTGTTTATAAC* *GTGTACATACATTAACTTATTACCTC* *ATTTTGTTATTTTTCGAAACAAAGCC* *CTGTGGAAGAAAATGGAAAACTTGA* *AGAAGCATTAAAGTCATTCTGTTAAG* *CTGCGTAAAgtggtcgtgtttatttgcttggggcg* *ggagtggagcaggaagagggattcccatcccccac* *atcctcttaagtcacttttcacgataccccaaatgaatg* *ggctccttggaagacaaaacttacatcttcccatgctc* *ccctgccggtttctgcagtggatcagatccattccaga* *tcactggcagctagtggtggcctgacttgaccctctgg* *ggtgtggcgaggcagctttcttcctctgcgcagcgctc* *ccgagtgttgccagc* *homology arms,* P2A, tdTomato, SV40 polyA signal, introns in lowercase; exons in uppercase.

CRediT authorship contribution statement

Peter J. Houweling: . **Vanessa Crossman:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. **Chrystal F. Tiong:** Data curation, Formal analysis, Writing – review & editing. **Chantal A. Coles:** Data curation, Formal analysis, Writing – review & editing. **Rhonda L. Taylor:** Methodology, Writing – review & editing. **Joshua S. Clayton:** Methodology, Writing – review & editing. **Alison Graham:** Formal analysis, Methodology, Writing – review & editing. **Katerina Vlahos:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Sara E Howden:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Kathryn N. North:** Conceptualization, Funding acquisition, Writing – review & editing.

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

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2024.103313.

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