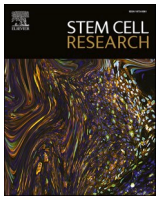
Stem Cell Research 69 (2023) 103079

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr



Lab Resource: Single Cell Line

Generation of an MTM1-mutant iPSC line (CRICKi008-A) from an individual with X-linked myotubular myopathy (XLMTM)

Liani G. Devito [a,1], Valentina M. Lionello [b,c,1], Francesco Muntoni [d], Francesco Saverio Tedesco [b,c,d,*], Lyn Healy [a,*]

[a] Human Embryo and Stem Cell Unit, The Francis Crick Institute, London, UK
[b] Stem Cells and Neuromuscular Regeneration Laboratory, The Francis Crick Institute, London, UK
[c] Department of Cell and Developmental Biology, University College London, London, UK
[d] Dubowitz Neuromuscular Centre, UCL Great Ormond Street Institute of Child Health and Great Ormond Street Hospital for Children, London, UK

A B S T R A C T

Centronuclear myopathies (CNMs) are a group of inherited rare muscle disorders characterised by the abnormal position of the nucleus in the center of the muscle fiber. One of CNM is the X-Linked Myotubular Myopathy, caused by mutations in the myotubularin (*MTM1*) gene (XLMTM), characterised by profound muscle hypotonia and weakness, severe bulbar and respiratory involvement. Here, we generated an induced pluripotent stem cell (iPSC) line from a patient with a severe form of XLMTM. Dermal fibroblasts were reprogrammed to pluripotency using a non-integrating mRNA-based protocol. This new MTM1-mutant iPSC line could facilitate disease-modelling and therapy development studies for XLMTM.

1. Resource table

Unique stem cell line identifier	*CRICKi008-A*
Alternative name(s) of stem cell line	*iFCI006*
Institution	*THE FRANCIS CRICK INSTITUTE*
Contact information of distributor	lyn.healy@crick.ac.uk liani.devito@crick.ac.uk saverio.tedesco@crick.ac.uk
Type of cell line	*iPSC*
Origin	*Human*
Additional origin info	*Age: 6 months*
Cell Source	*Dermal Fibroblast*
Clonality	*Clonal*
Method of reprogramming	*mRNA*
Genetic Modification	*NO*
Associated disease	*XLMTM caused by MTM1 mutation*
Gene/locus	*c.594C>G (p.Tyr198X) in MTM1 exon 8*
Date archived/stock date	*AUGUST 2022*
Cell line repository/bank	https://hpscreg.eu/cell-line/CRICKi008-A
Ethical approval	This study was approved by The London – West London & GTAC Research Ethics Committee (formerly known as the Hammersmith, Queen Charlotte's and Chelsea Research Ethics Committee). Fibroblasts were supplied by the MRC CNMD Biobank London (REC reference number 06/Q0406/33). Human cell work was conducted under the approval of the National Health Service (NHS) Health Research Authority Research Ethics Committee reference no. 13/LO/1826; Integrated Research Application System (IRAS) project no. 141,100.

(continued on next column)

(continued)

2. Resource utility

We report a patient-derived iPSC line for use as a disease-specific cellular model to further understand the pathogenesis of rare muscle disorder XLMTM caused by *MTM1* mutation. This new MTM1-mutant iPSC line could facilitate disease-modelling and therapy development studies for XLMTM (see Table 1).

3. Resource details

Centronuclear myopathies (CNMs) are a group of incurable muscle disorders characterized by the abnormal position of the nucleus in the center of the muscle fiber. The most severe CNM is the X-Linked Myotubular Myopathy, caused by mutations in the myotubularin (*MTM1*) gene (XLMTM, MIM #310400), characterised by profound muscle hypotonia and weakness (often with neonatal onset) severe bulbar and respiratory dysfunction and premature death. XLMTM remains

* Corresponding authors at: The Francis Crick Institute, 1 Midland Road, London NW1 1AT, UK (F.S. Tedesco).
E-mail addresses: saverio.tedesco@crick.ac.uk (F.S. Tedesco), lyn.healy@crick.ac.uk (L. Healy).
[1] Equal contribution.

https://doi.org/10.1016/j.scr.2023.103079
Received 7 February 2023; Accepted 18 March 2023
Available online 21 March 2023

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Microscopic photography	Normal morphology at passage 6–8	Fig. 1 panel A
Phenotype	Quantitative analysis Flow Cytometry	Staining of pluripotency markers: OCT4+, SSEA4+ and SSEA1-	Fig. 1 panel C
Genotype	CNV analysis: Karyostat assay (Thermo Scientific) with resolution >2 Mb for chromosomal gains and >1 Mb for chromosomal losses	Male/ female individual; no chromosome aberrations compared to reference dataset	Fig. 1 panel B
	STR analysis	All 16 sites matched	submitted in archive with journal
Mutation analysis	Sanger Sequencing	*State if heterozygous/ homozygous, type of mutation*	Fig. 1 panel B
Microbiology and virology	Mycoplasma	Mycoplasma testing by RT-PCR Negative	not shown but available with author
Differentiation potential	*In vitro* differentiation	Direct differentiation to three germ layers confirmed by immunostaining	Fig. 1 panel E
List of recommended germ layer markers	Spontaneous *In vitro* differentiation: EB formation followed by TaqMan™ hPSC Scorecard™ Panel, Fast 96-well (Cat. N. A15876)	Expression of markers (qPCR) of Endoderm, Mesoderm and Ectoderm germ layer confirmed by Scorecard	Fig. 1 Panel D

incurable, and several affected males present symptoms at birth and invariably develop respiratory failure in infancy or childhood. Here we report the generation and characterisation of a novel iPSC line derived from a male patient with a severe infantile form of XLMTM with a pathogenic *MTM1* mutation (Bachmann et al., 2017).

Patient dermal fibroblasts were obtained from UCL MRC Neuromuscular Disease Biobank and reprogrammed using the non-integrating mRNA-based protocol (StemRNA™ 3rd Gen Reprogramming Kit, REPROCELL) that combines non-modified RNA (NM-RNA) and microRNA technology. It contains six reprogramming factors, Oct4, Sox2, Klf4, cMyc, Nanog, Lin 28, and three immune evasion factors, E3, K3 and B18. Cells were reprogrammed in a feeder-free system according to the manufacturer's instructions. Colonies with a typical pluripotent stem cell morphology were individually and manually selected to establish clonal feeder-free iPSC lines.

Cells showed typical iPSC morphology after a few passages (Fig. 1A). Dideoxynucleotide sequencing (Sanger Sequencing) confirmed the presence of the pathogenic c.594C>G nonsense mutation in exon 8 of the MTM1 gene (Fig. 1B). Copy number variation analysis by chromosomal microarray confirmed the male sex of the individual with a benign partial chromosomal gain on chromosome Y (Fig. 1B). Stem cell identity of the CRICKi008-A was confirmed by the expression of pluripotency markers OCT4 and SSEA4, on Flow Cytometry analyses (Fig. 1C).

In vitro differentiation (direct and spontaneous) confirmed the cell line's ability to different onto all three germ layers (Fig. 1D and 1E). Identical genetic identity to the donor of the iPSC was confirmed by short tandem repeat (STR) profiling. Taken together, these results prove that we have successfully produced an iPSC line from a patient with a severe form of XLMTM.

4. Materials and methods

4.1. iPSC cell generation and expansion

Dermal fibroblasts were thawed at passage 4 and seeded them at a density of $5x10^4$/well in 2 wells of a 6-well plate coated with iMatrix (Stemgent). Cells were plated in Fibroblast Expansion Medium (DMEM (Gibco)/ Glutamax (Gibco)/ 10% Hyclone FBS (Thermo Scientific)) and cultured for 24 h in 37 °C, 5% CO_2 and 21% O_2. Then, on the first day of reprogramming, Day 0, the medium was switched to NutriStem medium (Stemgent), and cells were transferred to a hypoxic incubator at 37 °C, 5% CO_2 and 5% O_2.

Fibroblasts were reprogrammed 1-day post-seeding using the StemRNA™ 3rd Gen Reprogramming Kit (Stemgent) according to the manufacturer's instructions. Briefly, the NM-RNA cocktail was added to RNAiMAX transfection reagent (Gibco) and transfected into the cells for four consecutive days with medium change 6 h post-transfection.

iPSC-like colonies started to appear 9 days following the first transfections; they were manually picked and transferred to Matrigel-coated 6-well plates with mTeSR1 medium (StemCell Technologies) containing 10 μM Y-27362. The medium was changed after 24 h. Colonies were expanded by splitting at a 1:3 to 1:6 ratio every 4–6 days and maintained in a hypoxic incubator at 37 °C, 5% CO2 and 5% O2.

4.2. Pluripotency markers

We evaluated the pluripotency status of the CRICKi008-A line by Flow Cytometry using the BD Stemflow™ Human and Mouse Pluripotent Stem Cell Analysis Kit (BD) as per the manufacturer's instruction. Briefly, we detached the cells using Accutase (Sigma-Aldrich) and passed through a 70 μM cell strainer to eliminate cell clumps. Cells were then washed with DPBS (without Ca^2 Mg^2) (Thermo Fisher Scientific) and resuspended at 1×10^6 cells/ ml before adding the Live/ Dead staining (Thermo Fisher) for 30 min at room temperature. After another cell wash step with DPBS, cells were fixed in 4% paraformaldehyde (BD Stemflow Analyses kit component) for 20 min. For the permeabilization step, we used the 1X Perm/ Wash buffer (BD Stemflow Analyses kit component) for 10 min, followed by a wash in the same buffer. We incubated the cells with the antibodies (Table 2) for 30 min. We used DIVA software to analyse the cells and FlowJo to analyse the data.

4.3. Spontaneous differentiation into three germ layers

We tested the differentiation capacity of the CRICKi008-A line by spontaneous differentiation and embryoid body (EB) formation. Using the AggreWell™ 800 Microwell Plates, we seeded $5x10^6$/well in APEL 2 medium (STEMCELL Technologies) and cultured the EBs for 14 days in a hypoxic incubator at 37 °C, 5% CO2 and 5% O2.

The expression of the lineage-specific markers was assessed by TaqMan hPSC Scorecard Assay (Thermo Scientific). Briefly, we isolated RNA using the QIAgen RNeasy mini kit and measured concentration by a Nanodrop. We used the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher) for cDNA preparation. We analysed the data using the hPSC Scorecard™ – Analysis group (Thermo Fisher).

4.4. Direct differentiation into three germ layers

For direct differentiation, we used the STEMdiff™ Trilineage Differentiation Kit (STEMCELL Technologies) as per manufacturer's instructions. We evaluated the differentiation potential of the CRICKi008-A line by immunostaining for lineage-specific markers on Day 5 (Mesoderm and Endoderm) and Day 7 (Ectoderm) as previously described (Devito et al., 2021). Differentiated cells were washed twice in DPBS (Ca^2 Mg^2) (Thermo Fisher Scientific) before fixation with 3.7% paraformaldehyde (Sigma-Aldrich) for 20 min at room temperature.

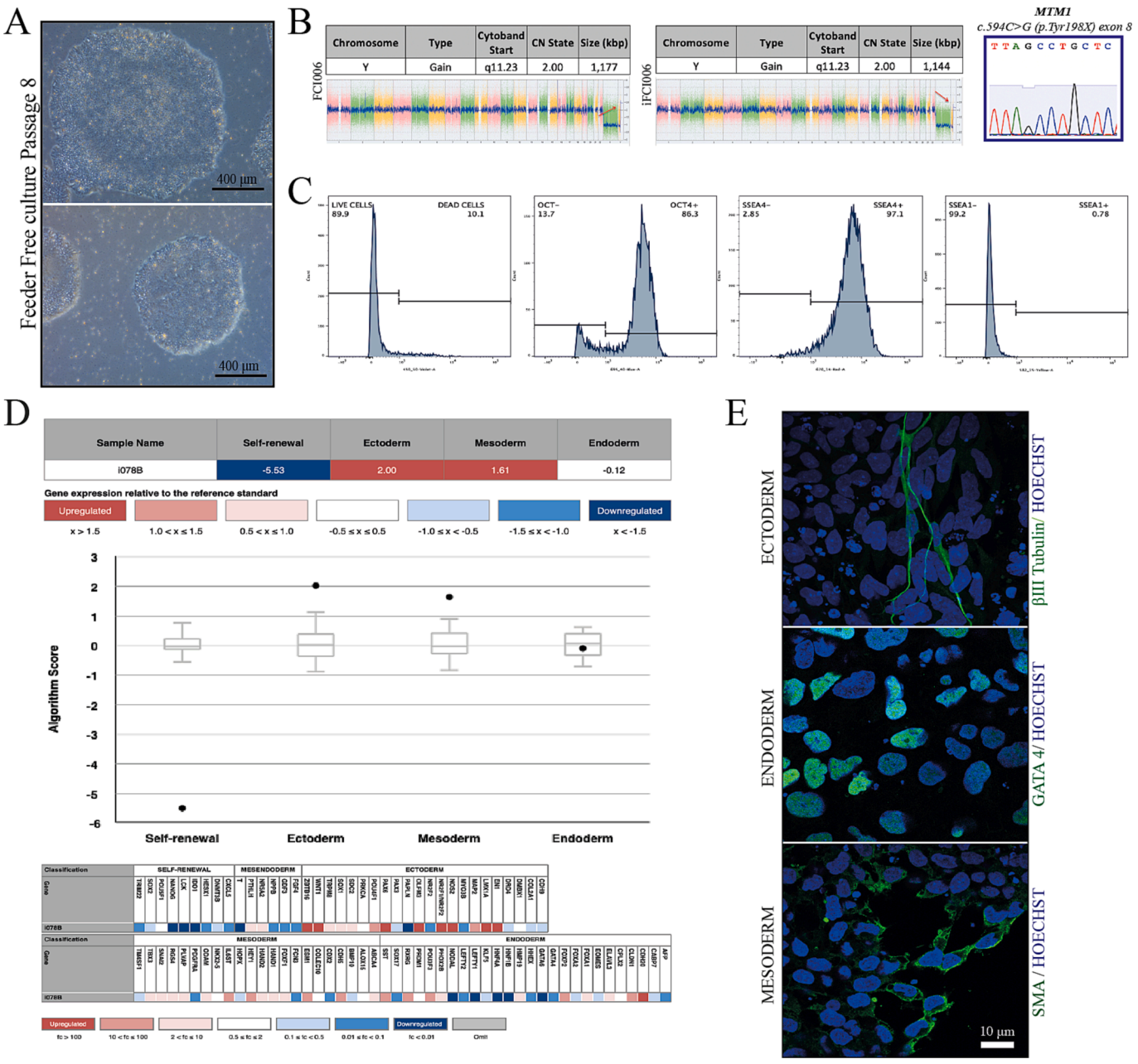


Fig. 1.

Cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) for 5 min at room temperature, then incubated with primary antibodies (Table 2) overnight at 4 °C. The following day, cells were washed twice with DPBS and incubated with secondary antibodies (Table 2) for 30 min at room temperature.

4.5. Dideoxy nucleotide sequencing

We performed PCR amplification (primers listed in Table 2) using the Q5 High-Fidelity 2X Master-Mix (BioLabs) on cell line genomic DNA extracted using the QIAamp DNA micro Kit (Qiagen). Then we purified the PCR product using the Monarch PCR and DNA cleanup Kit (BioLabs). Finally, we sent the samples for Sanger sequencing to Source Biosciences (UK), and analysed the data using SnapGene software.

4.6. Chromosomal microarray

Using genomic DNA of the iPSC lines, Thermo Scientific (USA) performed the KaryoStat assay (Thermo Scientific, USA), an array comparative genomic hybridization (CGH).

4.7. Short tandem repeat (STR) profiling

The Cell Services, a Science Technology Platform (STP) within the Francis Crick Institute, performed the STR profiling on DNAs from the parental sample and iPSC line using the Powerplex 16 HS System (Promega). All lines were sent regularly for STR profiling (every 3 passages) since reprogramming started.

4.8. Mycoplasma detection test

The Cell Services (STP) confirmed the absence of mycoplasma

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry				
	Antibody	Dilution/Amount of staining	Company Cat #	RRID
Pluripotency Markers	BD PharmingenTM Alexa Fluor® 647 Mouse anti-SSEA-4	20 μL per sample (5x10^5 to 1x 0^6 cells)	BD #560477	AB_2869350
	BD PharmingenTM PE Mouse anti-SSEA-1	20 μL per sample (5x10^5 to 1x 0^6 cells)	BD #560477	AB_2869350
	BD PharmingenTM PerCP-CyTM5.5 Mouse anti-Oct3/4	20 μL per sample (5x10^5 to 1x 0^6 cells)	BD #560477	AB_2869350
Differentiation Markers	Rabbit anti-GATA-4	1:500	Cell Signaling Technology #36966	AB_2799108
	Mouse anti-βIII-tubulin	1:100	Invitrogen # MA1-118	AB_2536829
	Mouse anti smooth muscle actin SMA	1:100	Sigma #A5228	AB_262054
	Donkey anti-goat FITC IgG	1:100	Jackson Immunoresearch #705-095-147	AB_2340401
	Donkey anti-rabbit Alexa Fluor	1:500	Thermofisher #A-21206	AB_2535792
	Goat anti-rabbit Alexa Fluor	1:500	Thermofisher #A-11029	AB_2534088
Primers	Target	Forward/Reverse primer (5′-3′)		Size of Band
Targeted mutation analysis	*MTM1* (F)	CCC TGT GGT CAA AGA GGC AT		300 bp
	MTM1 (R)	TCC AAA AGG ACC GTT TCT CTA AGT		

contamination using PCR amplification using the Universal Mycoplasma Detection Kit (ATCC 30–1012 K) for PCR amplification. Cells were regularly sent for Mycoplasma testing (every 3 passages) since reprogramming started.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Liani G Devito reports financial support was provided by The Francis Crick Institute.

Acknowledgments

The authors wish to thank the patient's family, Mr Pierpaolo Ala (MRC Centre Neuromuscular Biobank Research Coordinator) and the MRC Centre for Neuromuscular Diseases Biobank London, supported by the National Institute for Health Research Biomedical Research Centres at Great Ormond Street Hospital for Children NHS Foundation Trust and University College London Hospitals NHS Foundation Trust and UCL, for providing fibroblasts. We thank the Francis Crick Institute Genomics Equipment Park, Cell Services STPs and HESCU LRS Ilenia D'Angelo. This work was supported by: The Francis Crick Institute, which receives its funding from Cancer Research UK (CC0199), the UK Medical Research Council (CC0199) and the Wellcome Trust (CC0199). For the purpose of Open Access, the author has applied a CC BY public copyright licence to any Author Accepted Manuscript version arising from this submission. VML and FST acknowledge funding by AFM-Telethon (23782). FST acknowledges support by the European Research Council (759108 – HISTOID) and the NIHR (CL-2018-18-008; the views expressed are those of the authors and not necessarily those of the National Health Service, the NIHR, or the Department of Health).

References

Bachmann, C., Jungbluth, H., Muntoni, F., Manzur, A.Y., Zorzato, F., Treves, S., 2017. Cellular, biochemical and molecular changes in muscles from patients with X-linked myotubular myopathy due to MTM1 mutations. Human Mol. Genetics 26, 2.

Devito, L.G., Healy, L., Mohammed, S., Guillemot, F., Dias, C., 2021. Generation of an iPSC line (CRICKi001-A) from an individual with a germline SMARCA4 missense mutation and autism spectrum disorder. Stem Cell Res. 53, 102304.