Stem Cell Research 65 (2022) 102954

Contents lists available at ScienceDirect

Stem Cell Research

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

Lab Resource: Multiple Cell Lines

Generation of FOUR iPSC lines (CRICKi004-A; CRICKi005-A; CRICKi006-A, CRICKi007-A) from Spinal muscle atrophy patients with lower extremity dominant (SMALED) phenotype

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A B S T R A C T

Spinal muscular atrophy with lower extremity dominant (SMALED) is a hereditary neuromuscular disorder characterized by degeneration of spinal cord motor neurons resulting in lower limbs muscle weakness and paralysis. Mutations in *DYNC1H1*, which encodes *BICD2*, a multifunctional adaptor for microtubule motor proteins, cause the disorder. Here, we generated four induced pluripotent stem cell (iPSC) lines from patients with SMALED. Dermal fibroblasts were obtained from the MRC neuromuscular disease biobank and reprogrammed using non-integrating mRNA-based protocol. Characterization of the four iPSC lines included karyotyping and Sanger sequencing, while the expression of associated markers confirmed pluripotency and differentiation potential.

Resource table

Unique stem cell lines identifier	CRICKi004-A	CRICKi005-A	CRICKi006-A	CRICKi007-A
Alternative name(s) of stem cell lines	*iFCI008*	*iFCI004*	*iFCI005*	*iFC009*
Institution	*The Francis Crick Institute*			
Contact information of distributor	lyn.healy@crick.ac.uk, liani.devito@crick.ac.uk			
Type of cell lines	*iPSC*			
Origin	*Human*			
Additional origin info required for human iPSC	*FCI008* *Age: 3* *Sex: female*	*FCI004* *Age: 40* *Sex: male*	*FCI005* *Age: 3* *Sex: male*	*FCI009* *Age: 11* *Sex: female*
Cell Source	*Dermal Fibroblast*			
Clonality	*Clonal*			
Method of reprogramming	*mRNA*			
Genetic Modification	*NO*			
Type of Genetic Modification	*N/A*			
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	*N/A*			
Associated disease	Spinal muscular atrophy lower extremity-predominant autosomal dominant (SMALED)			
Gene/locus	*FCI008* *DYNC1H1* Homozygous mutation c.1195A>G p.R399G	*FCI004* *DYNC1H1* Heterozygous mutation c.1195A>G p.R399G	*FCI005* *DYNC1H1* De novo heterozygous mutation c.1012G>A, p.D338N	*FCI009* *BICD2* De novo heterozygous mutation c.565A>T, p.I189F

(*continued on next page*)

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https://doi.org/10.1016/j.scr.2022.102954
Received 20 October 2022; Accepted 27 October 2022
Available online 28 October 2022

Resource table (*continued*)

Unique stem cell lines identifier	CRICKi004-A	CRICKi005-A	CRICKi006-A	CRICKi007-A
Date archived/stock date	*DECEMBER 2021*			
Cell line repository/bank	https://hpscreg.eu/cell-line/CRICKi004-A	https://hpscreg.eu/cell-line/CRICKi005-A	https://hpscreg.eu/cell-line/CRICKi006-A	https://hpscreg.eu/cell-line/CRICKi007-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).			

1. Resource utility

We report four patient-derived iPSC lines available for use as a disease-specific cellular model to further understand Spinal muscle atrophy, lower extremity dominant (SMALED) phenotype.

2. Resource details

Spinal muscular atrophy lower extremity-predominant autosomal dominant (SMALED) is a hereditary disorder characterized by nonprogressive or early-onset muscle weakness affecting the proximal muscles of the lower extremities (quadriceps) resulting in atrophy and paralysis. Mutations in *DYNC1H1*, which encodes a microtubule motor protein in the dynein-dynactin complex and one of its cargo adaptors, *BICD2*, are a common cause of the disorder (Harms et al, 2010; Scoto et al., 2015, Rossor et al., 2015).

Here we report the generation and validation of four iPSC lines derived from four patients with SMALED.

We obtained patient dermal fibroblast from the 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, together with three immune evasion factors E3, K3 and B18. Cells were reprogrammed in a feeder-free system according to manufacturer's instruction. 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). The stem cell identity of CRICKi004-A; CRICKi005-A; CRICKi006-A, CRICKi007-A were confirmed by expression of pluripotency markers *OCT4* and *SSEA4*, on Flow Cytometry analyses (Fig. 1C).

We confirmed the mutation by Dideoxynucleotide sequencing (Sanger Sequencing) (Fig. 1B). Copy number variation analysis by chromosomal microarray indicated the sex of the individual (F or M) with no chromosomal aberrations (Fig. 1B).

In vitro differentiation (direct and spontaneous) confirmed the ability to different to 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.

3. Materials and methods

3.1. iPSC cell generation and expansion

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

Cells were reprogrammed 1-day post-seeding, using the StemRNA™ 3rd Gen Reprogramming Kit (Stemgent) according to the manufacturer's instructions. Briefly, 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 show 10 days post first day of transfections. Those with appropriate morphology were manually picked and transferred to Matrigel (Corning, hESC qualified) coated 6-well plates with mTeSR1 medium (STEMCELL Technologies) containing 10 μM Y-27362. Media was changed after 24 h. Colonies were expanded by splitting at 1:3 to 1:6 ratio every 4–6 days and maintained in a hypoxic incubator at 37 °C, 5 % CO2 and 5 % O2 (Table 1).

3.2. Pluripotency markers

All lines were evaluated for their pluripotency potential by Flow Cytometry using the BD Stemflow™ Human and Mouse Pluripotent Stem Cell Analysis Kit (BD) as per manufacturer's instruction. Briefly, cells were detached using Accutase (Sigma-Aldrich) and passed through a 70 μM cell strainer to eliminate cell clumps. We washed the cells with DPBS (without Ca^2 Mg^2) (Thermo Fisher Scientific) and resuspended at 1×10^6 cells/ ml prior to add the Live/ Dead staining (Thermo Fisher) for 30 min at room temperature. Cells were then washed once with DPBS prior to fixation with 4 % paraformaldehyde (BD Stemflow Analyses kit component) for 20 min. Cells were permeabilized with 1X Perm/ Wash buffer (BD Stemflow Analyses kit component) for 10 min then incubated with the antibodies (Table 2) for 30 min. We used DIVA software to analyse the cells and FlowJo to analyse the data.

3.3. iPSC differentiation into three germ layers

All iPSC lines were spontaneously differentiated using AggreWell™ 800 Microwell Plates in STEMdiff™ APEL 2 medium (STEMCELL Technologies) per manufacturer's instructions and expression of the lineage-specific markers was assessed by TaqMan hPSC Scorecard Assay (Thermo Scientific). Briefly, RNA was isolated using the QIAgen RNeasy mini kit and concentration was measured 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 hPSC Scorecard™ – Analysis group (Thermo Fisher).

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

3.4. Dideoxynucleotide 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

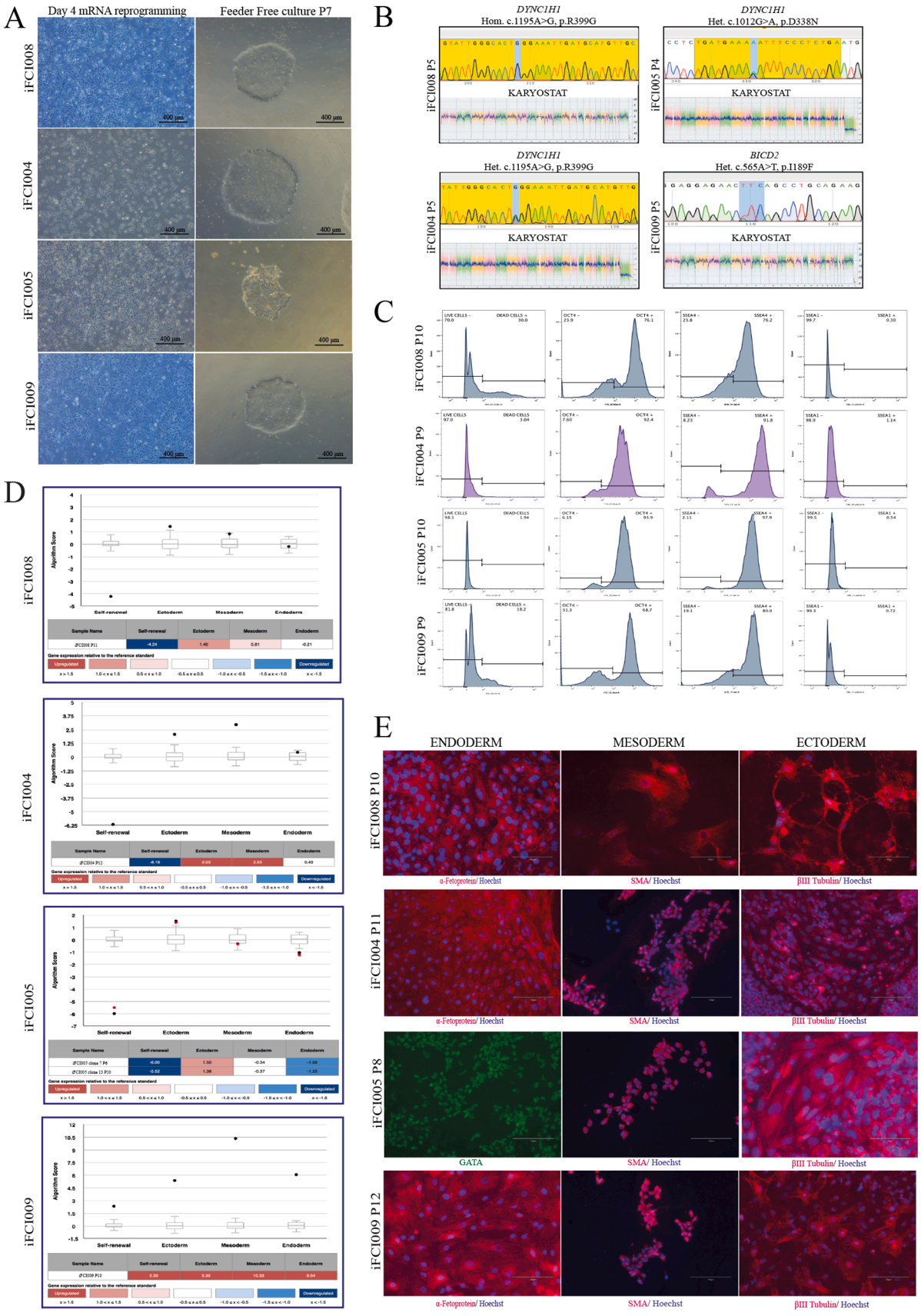
A
Day 4 mRNA reprogramming
Feeder Free culture P7
iFCI1008
iFCI1004
iFCI1005
iFCI1009
400 µm
B
DYNC1H1
Hom. c.1195A>G, p.R399G
DYNC1H1
Het. c.1012G>A, p.D338N
DYNC1H1
Het. c.1195A>G, p.R399G
BICD2
Het. c.565A>T, p.I189F
iFCI1008 P5
iFCI1005 P4
iFCI1004 P5
iFCI1009 P5
KARYOSTAT
C
iFCI1008 P10
iFCI1004 P9
iFCI1005 P10
iFCI1009 P9
D
iFCI1008
iFCI1004
iFCI1005
iFCI1009
Algorithm Score
Self-renewal
Ectoderm
Mesoderm
Endoderm
E
ENDODERM
MESODERM
ECTODERM
iFCI1008 P10
iFCI1004 P11
iFCI1005 P8
iFCI1009 P12
α-Fetoprotein/ Hoechst
SMA/ Hoechst
βIII Tubulin/ Hoechst
GATA

Fig. 1.

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	Specific how many sites tested, and if matched or not	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
Donor screening	HIV1 and 2; Hepatitis B, Hepatitis C; Cytomegalovirus; *T*-lymphoytopic; Epstein-Barr	Negative	not shown but available with author

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 (5×10^5 to 1x 0^6 cells)	BD #560477	AB_2869350
	BD PharmingenTM PE Mouse anti-SSEA-1	20 μL per sample (5×10^5 to 1x 0^6 cells)	BD #560477	AB_2869350
	BD PharmingenTM PerCP-CyTM5.5 Mouse anti-Oct3/4	20 μL per sample (5×10^5 to 1x 0^6 cells)	BD #560477	AB_2869350
Differentiation Markers	Goat anti-GATA-4	1:100	R&D System #AF2606	AB_2232177
	Mouse anti-βIII-tubulin	1:100	Sigma #T5076	AB_532291
	Mouse anti smooth muscle actin SMA	1:100	Sigma #A5228	AB_262054
	Mouse anti-Alpha fetoprotein	1:100	Sigma #A8452	AB_258392
Secondary antibodies	Donkey anti-mouse Rhodamine IgG	1:100	Jackson Immunoresearch #715-295-150	AB_2340831
	Donkey anti-goat FITC IgG	1:100	Jackson Immunoresearch #705-095-147	AB_2340401
	Donkey anti-rabbit FITC IgG	1:100	Jackson Immunoresearch #711-095-152	AB_2315776
	Donkey anti-mouse Alexa Fluor IgM	1:100	Jackson Immunoresearch #715-545-140	AB_2340845

Primers	**Target**	**Forward/Reverse primer (5′-3′)**	**Size of Band**
Targeted mutation analysis	*BICD2* F1	GCTGCTCATACCTGCCTTTC	275
	BICD2 R1	CCCAAGACTCACCTGGTTCT	275
	DYNC1H1 F1	AGGTCTAAAACAGGCTTTGGA	388
	*DYNC1H1 R*1	ACTCATCATCCCAAGTCTGAAA	388

(UK), and analysed the data using SnapGene software.

3.5. 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). All lines were sent at Passage number 7.

3.6. 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.

3.7. Mycoplasma detection test

The Cell Services (STP) confirmed the absence of mycoplasma contamination using PCR amplification using the Universal Mycoplasma Detection Kit (ATCC 30-1012K) for PCR amplification. Cells were regularly sent for Mycoplasma testing (every 3 passages) since reprogramming started.

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.

Acknowledgments

The authors wish to thank the patient and family. We thank the Francis Crick Institute Genomics Equipment Park and Cell Services STPs.

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.

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