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Lab Resource: Multiple Cell Lines

Generation of genetically matched hiPSC lines from two mosaic facioscapulohumeral dystrophy type 1 patients

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ABSTRACT

Facioscapulohumeral dystrophy type 1 (FSHD1) is caused by contraction of the D4Z4 repeat array on chromosome 4q resulting in sporadic misexpression of the transcription factor DUX4 in skeletal muscle tissue. In ~4% of families, *de novo* D4Z4 contractions occur after fertilization resulting in somatic mosaicism with control and FSHD1 cell populations present within the same patient. Reprogramming of mosaic fibroblasts from two FSHD1 patients into human induced pluripotent stem cells (hiPSCs) generated genetically matched control and FSHD1 hiPSC lines. All hiPSC lines contained a normal karyotype, expressed pluripotency genes and differentiated into cells from the three germ layers.

Resource utility

The newly generated genetically matched hiPSC lines from the two mosaic FSHD1 patients are useful for disease modelling, next generation sequencing and drug testing.

Resource details

Facioscapulohumeral dystrophy type 1 (FSHD1) is caused by a contraction of the D4Z4 macrosatellite repeat array located on chromosome 4q. Healthy individuals have a D4Z4 repeat array size of 8–100 units, while patients with FSHD1 carry a contracted repeat of 1–10 units (van der Maarel et al., 2012). A D4Z4 repeat array contraction causes sporadic *DUX4* misexpression in skeletal muscle leading to muscle weakness and wasting in patients. In some families (4%) *de novo* contractions of the D4Z4 repeat array occur post-fertilization resulting in a mosaicism with healthy and FSHD1 cell populations present within a single individual (Lemmers et al., 2004). With the reprogramming of mosaic fibroblasts into clonal hiPSC colonies it is possible to separate cell populations and to generate control and disease hiPSC lines with an identical genetic background. We selected two mosaic FSHD1 fibroblast lines with a balanced ratio of control and FSHD1 cell populations and reprogrammed the mosaic fibroblasts into hiPSCs using synthetic RNA (Yoshioka et al., 2013). Single colonies were picked and analyzed for D4Z4 repeat array size using pulsed field gel electrophoresis followed by Southern blot analysis (Lemmers, 2017). We detected a contracted D4Z4 repeat array in 33% and 25% of hiPSC clones generated from patient 1 and patient 2, respectively (data not shown). We next selected one control and one FSHD1 line from each patient for complete characterization (Tables 1 and 2). In 0162-FSHD04 and 0163-FSHD04 we detected the FSHD1-sized D4Z4 repeat array, while in 0162-CTRL05 and 0163-CTRL05 we detected the normal-sized D4Z4 repeat array confirming the successful separation of control and FSHD1 cell populations after reprogramming (Fig. 1A). All lines showed a typical hiPSC morphology (Fig. 1B) and were negative for mycoplasma (Supplementary Fig. S1A). Immunofluorescence analysis of pluripotency genes revealed that all lines expressed OCT3/4, NANOG and SSEA4 (Fig. 1C). Quantification of the expression levels of *OCT4*, *SOX2* and *NANOG* in 0162 and 0163 lines showed a comparable expression to control hiPSC line 114–1 which was previously published (Buijsen et al., 2018). All hiPSC lines in this study showed a similar expression level of pluripotency genes between lines and patients, while the fibroblasts from which the hiPSC lines originated demonstrated no expression (Fig. 1D). We next analyzed the hiPSC lines for unbalanced

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Table 1
Summary of lines.

hiPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus (# 4qA D4Z4 repeats)	Disease
LUMC0162iCTRL05	0162-CTRL05	Male	53	Caucasian	45	Control
LUMC0162iFSHD04	0162-FSHD04	Male	53	Caucasian	3	FSHD1
LUMC0163iCTRL05	0163-CTRL05	Male	60	Caucasian	43	Control
LUMC0163iFSHD04	0163-FSHD04	Male	60	Caucasian	2	FSHD1

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Brightfield microscopy	Normal morphology	Fig. 1, panel B
Phenotype	Qualitative analysis by immunofluorescence staining	Positive staining of pluripotency markers: OCT3/4, NANOG and SSEA4	Fig. 1, panel C
	Quantitative analysis by RT-qPCR	Expression of pluripotency markers OCT4, SOX2 and NANOG	Fig. 1, panel D
Genotype	GSAMD24 v1 Illumina Infinium SNP array 700 k	CNV report resolution 50 kb: No major copy number variations or allelic changes	Fig. 1, panel E
Identity	GSAMD24 v1 Illumina Infinium SNP array 700 k	GSA array	Summarized data in Supplementary Fig. S1, panel B
		Fibroblasts and hiPSCs have > 99,99% identical SNPs	Data available upon request
Mutation analysis (IF APPLICABLE)	Pulsed field gel electrophoresis / Southern blot analysis	0162-CTRL05: D4Z4 4qA 155 kb (45 repeats) 0162-FSHD04: D4Z4 4qA 15 kb (3 repeats) 0163-CTRL05: D4Z4 4qA 147 kb (43 repeats) 0163-FSHD04: D4Z4 4qA 13 kb (2 repeats)	Fig. 1, panel A
Microbiology and virology	Mycoplasma	Mycoplasma testing by luminescence: Negative	Supplementary Fig. S1, panel A
Differentiation potential	Qualitative analysis by immunofluorescence staining	Positive staining of germ layer markers Vimentin (mesoderm), PAX6 (ectoderm) and FOXA2 (endoderm)	Fig. 1, panel F

chromosomal abnormalities using a Global Screening Array (GSA v1 Illumina Inc.) carrying probes for 700 k single nucleotide polymorphisms (SNPs). We quantified for each SNP the relative signal intensities and detected no copy number abnormalities (resolution: ~50 kb) (Fig. 1E). Comparing 700 k SNPs of hiPSC lines with the original fibroblasts showed an overlap of at least > 99,9% (Supplementary Fig. S1B) demonstrating that patient 1 fibroblasts were identical to 0162-CTRL05 and 0162-FSHD04 and that patient 2 fibroblasts were identical to 0163-CTRL05 and 0163-FSHD04. Finally, we induced spontaneous differentiation and determined the formation of cells from the three germ layers. In all hiPSC lines we detected cells positive for Vimentin (mesoderm), PAX6 (ectoderm) and FOXA2 (endoderm) showing the capacity of the hiPSCs to differentiate towards cells from the three germ layers (Fig. 1F).

Materials and methods

Ethical statement

Fibroblasts previously obtained from anonymized human skin biopsies from individuals with FSHD1 were provided by the Fields Center for FSHD Research biorepository and utilized in this study to create hiPSC lines (RSRB00059324). This study was performed in accordance and approval of the LUMC scientific ethical committee.

Generation of hiPSCs

Fibroblasts were reprogrammed using synthetic RNA with the ReproRNA™-OKSGM kit (STEMCELL Technologies) according to the manufacturer's instructions. After reprogramming single colonies were picked and expanded in TESR-E8 medium (STEMCELL Technologies). Confluent cultures were passaged with gentle cell dissociation reagent on Vitronectin XF coated plates (STEMCELL Technologies).

Pulsed field electrophoresis and southern blot analysis

D4Z4 repeat array size was determined with pulsed field gel electrophoresis followed by Southern blot analysis according to a protocol previously established in our laboratory (Lemmers, 2017). For each hiPSC line, an agarose block containing 1×10^6 hiPSCs was digested with *Eco*RI and *Hind*III restriction enzymes (Thermo Scientific). The D4Z4 repeat array was detected with a radioactive labelled p13E-11 DNA probe recognizing the p13E-11 region proximal of the D4Z4 repeat array. The D4Z4 hybridization pattern at the ancestral fibroblasts served as a reference.

Spontaneous differentiation

hiPSCs were passaged on glass coverslips coated with Matrigel (Corning) and spontaneous differentiation was induced with the STEMdiff™ Trilineage Differentiation Kit (STEMCELL Technologies). After 5 days (endoderm and mesoderm) or 7 days (ectoderm) cells were fixed with 2% paraformaldehyde (PFA) for 30 min at room temperature and used for immunofluorescence staining.

Immunofluorescence staining

PFA-fixed hiPSCs on coverslips were permeabilized with 0.1% Triton X-100, blocked for 1 h in 4% normal swine serum (NSS, DAKO)/PBS, washed once with PBS and incubated for 1 h with primary antibodies (Table 3) diluted in blocking buffer. Coverslips were next washed 3 times for 10 min with washing buffer (0.05% Tween in PBS) followed by an incubation step with secondary antibodies in blocking buffer for 1 h. Finally, coverslips were washed 3 times for 10 min with washing buffer, incubated with DAPI (1:200, Thermo Scientific) in PBS for 5 min and imaged with a Leica TCS SP8 microscope.

RNA isolation and quantitative RT-PCR

RNA was extracted using the miRNeasy mini kit including a DNase I treatment (Qiagen). 800 ng of RNA was next reverse transcribed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Fisher). The resulting cDNA was diluted 50× and used in a qRT-PCR reaction consisting of 7.5 µl SybrGreen (Bio-Rad) and 10 pmol of

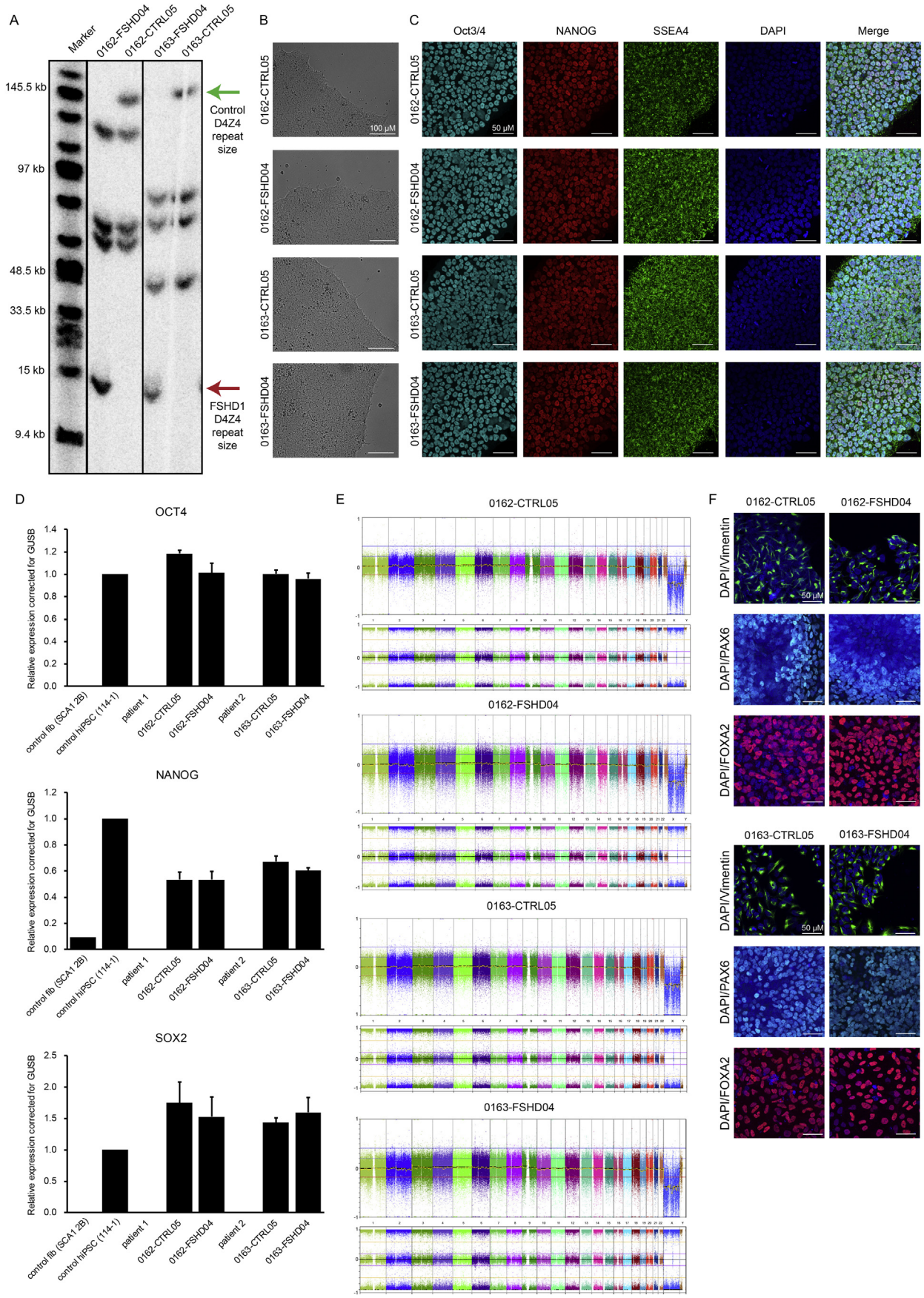

Fig 1. Characterization of hiPSC lines. (A) Detection of the D4Z4 repeat with pulsed field gel electrophoresis and Southern blotting. Arrows indicate the FSHD1-sized repeat (red) or the control-sized repeat (green). (B) Representative light microscope images of hiPSCs. (C) Immunofluorescence staining of OCT3/4, NANOG, SSEA4. Nuclei were stained with DAPI. (D) qRT-PCR analysis of OCT4, *NANOG* and *SOX2* expression in primary fibroblasts and generated hiPSCs. Data are normalized for *GUSB*. (E) Analysis of the copy number of 700k single nucleotide polymorphisms using the Global Screening Array (GSA v1 Illumina Inc.).

Table 3
Reagents details.

Antibodies used for immunocytochemistry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Mouse IgG2b anti-Oct-3/4	1:100	Santa Cruz Biotechnology Cat# sc-5279, RRID:AB_628051
	Mouse IgG1 anti-Nanog	1:150	Santa Cruz Biotechnology Cat# sc-293,121, RRID:AB_2665475
	Mouse IgG3 anti-SSEA-4	1:30	BioLegend Cat# 330402, RRID:AB_1089208
Differentiation markers	Mouse IgG1 anti-Vimentin (mesoderm)	1:50	Sigma-Aldrich Cat# V6630, RRID: AB_477627
	Rabbit anti-PAX6 (ectoderm)	1:200	Cell Signaling Technology Cat# 60433, RRID:AB_2797599
	Rabbit anti-FOXA2 (endoderm)	1:100	Millipore Cat# 07–633, RRID:AB_390153
Secondary antibodies	Goat anti-Mouse IgG2b Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	1:250	Thermo Fisher Scientific Cat# A-21242, RRID:AB_2535811
	Goat anti-Mouse IgG3 Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:250	Thermo Fisher Scientific Cat# A-21151, RRID:AB_2535784
	Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	1:250	Thermo Fisher Scientific Cat# A-21124, RRID:AB_2535766
	Donkey anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	1:250	Thermo Fisher Scientific Cat# A-31573, RRID:AB_2536183

Primers		
	Target	Forward/Reverse primer (5′-3′)
Pluripotency markers (qPCR)	OCT4	TGTACTCCTCGGTCCCTTTC/ TCCAGGTTTTCTTTCCCTAGC
	NANOG	CAGTCTGGACACTGGCTGAA/ CTCGCTGATTAGGCTCCAAC
	SOX2	GCTAGTCTCCAAGCGACGAA/ GCAAGAAGCCTCTCCTTGAA
Housekeeping gene (qPCR)	GUSB	CTCATTTGGAATTTTGCCGATT/ CCGAGTGAAGATCCCCTTTTTA

forward and reverse primers (Table 3). Reactions were analyzed on a CFX 96 machine (Bio-Rad).

Genomic DNA isolation and global screening array

Genomic DNA was extracted using a standard high salt protocol followed by purification with the DNA Clean & Concentrator kit (Zymo Research). 200 ng of genomic DNA was next loaded on a Global Screening Array (GSA) (Illumina) according to the manufacturer's instructions. Generated GSA manifest files were analyzed with GenomeStudio software and final reports were visualized using Nexus Discovery (BioDiscovery El Segundo).

Mycoplasma detection

Cultures were tested for mycoplasma with MycoAlert Mycoplasma detection kit (Lonza, Walkersville, MD) according to the manufacturer's instructions.

Key resource table

Unique stem cell lines identifier	LUMCi011-A LUMCi011-B LUMCi012-A LUMCi012-B
Alternative names of stem cell lines	LUMCi011-A: LUMC0162iCTRL05 and 0162-CTRL05 LUMCi011-B: LUMC0162iFSHD04 and 0162-FSHD04 LUMCi012-A: LUMC0163iCTRL05 and 0163-CTRL05 LUMCi012-B: LUMC0163iFSHD04 and 0163-FSHD04
Institution	Leiden University Medical Center (LUMC), Leiden, The Netherlands
Contact information of distributor	Dr. J.C. de Greef, j.c.de_greef@lumc.nl
Type of cell lines	hiPSCs
Origin	Human
Cell source	Skin fibroblasts
Clonality	Clonal
Method of reprogramming	Non-integrating synthetic RNA
Multiline rationale	Genetically matched control/disease
Gene modification	Yes
Type of modification	Heredity
Associated disease	FSHD1
Gene/locus	Patient1: 4qA, 4q35 D4Z4 repeats: 45 (control) / 3 (FSHD1) Patient2: 4qA, 4q35 D4Z4 repeats: 43 (control) / 2 (FSHD1)
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	November 2018
Cell line repository/bank	https://hpscreg.eu/cell-line/LUMCi011-A https://hpscreg.eu/cell-line/LUMCi011-B https://hpscreg.eu/cell-line/LUMCi012-A https://hpscreg.eu/cell-line/LUMCi012-B
Ethical approval	RSRB00059324, Research Subjects Review Board, University of Rochester.

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Declaration of Competing Interest

The authors declare to have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101560.

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