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Lab Resource: Multiple Cell Lines

Generation of two Duchenne muscular dystrophy patient-specific induced pluripotent stem cell lines DMD02 and DMD03 (MUNIi001-A and MUNIi003-A)

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A B S T R A C T

Duchenne muscular dystrophy (DMD) affects 1:3500–5000 newborn boys and manifests with progressive skeletal muscle wasting, respiratory failure and eventual heart failure. Symptoms show different onset from patients' childhood to the second decade of age. We reprogrammed fibroblasts from two independent DMD patients with a complete loss of dystrophin expression, carrying deletions of exons 45–50 and 48–50. The resulting hiPSCs show expression of pluripotency markers (NANOG, OCT4, SSEA4), differentiation capacity into all three germ layers, normal karyotype, genetic identity to the originating parental fibroblasts and the patient-specific dystrophin mutation.

Resource utility

Two human induced pluripotent stem cell (hiPSC) lines carrying mutations in the *DMD* gene were generated to study the development of Duchenne muscular dystrophy (DMD) *in vitro*, from a perspective of stem cell damage and in perspective to the pathophysiology of the resulting hiPSC-derived cardiomyocytes (CMs) and skeletal myotubes.

Resource details

DMD is an X-linked muscular degenerative disease which causes progressive weakness and loss of ambulation in the early teen years (Emery et al., 2015). Despite the progress of clinical care, heart failure or respiratory failure lead to death by the end of the 2nd decade of life.

DMD is caused by mutations in the *DMD* gene coding dystrophin protein leading to destabilization of the dystrophin glycoprotein complex (DGC), with sarcolemmal instability, cell death, fibrosis and lipid expansion in skeletal and cardiac muscles. Up to date, there is no curative therapy for this condition. DMD-associated dilated cardiomyopathy (DCM) is a major cause of mortality in affected patients and requires dedicated investigation and realistic models. The most common DMD model is the mdx mouse(Sicinski et al., 1989), although the cardiac symptoms are mild and progress slowly, compared to humans relative to lifespan. Therefore, the mdx model carries major limitations in cardiac disease modelling. Similarly, large animal models like golden retriever muscular dystrophy (GRMD), canine X-linked muscular dystrophy (CXMD), genetically engineered swine model, and feline muscular dystrophy (HFMD) have severe limitations coming from the animal physiology and variable cardiac involvement(Emery et al., 2015).

To investigate the human DMD cardiac phenotype using a cellular model, we generated patient-specific hiPSCs lines that were subsequently differentiated into cardiomyocytes. The informed consent forms were obtained according to the Ethical Committee and Helsinki declaration. Skin/muscle biopsies were harvested from two independent DMD boys (10- and 14-years old). From the biopsies, we derived two hiPSC lines harbouring deletions in *DMD* gene spanning exons 45–50 (DMD02, MUNIi001-A) and exons 48–50 (DMD03, MUNIi003-A). Individual clones were chosen empirically based on morphology. Selected clones from both lines tested positive for pluripotent markers alkaline phosphatase, Nanog, Oct3/4, TRA1–81 and SSEA4 (Fig. 1 A) and their expression pattern was comparable to wild type (WT) hiPSC. Quantitative analysis of pluripotency markers' mRNAs revealed similar expression of Oct4 and Nanog in both DMD

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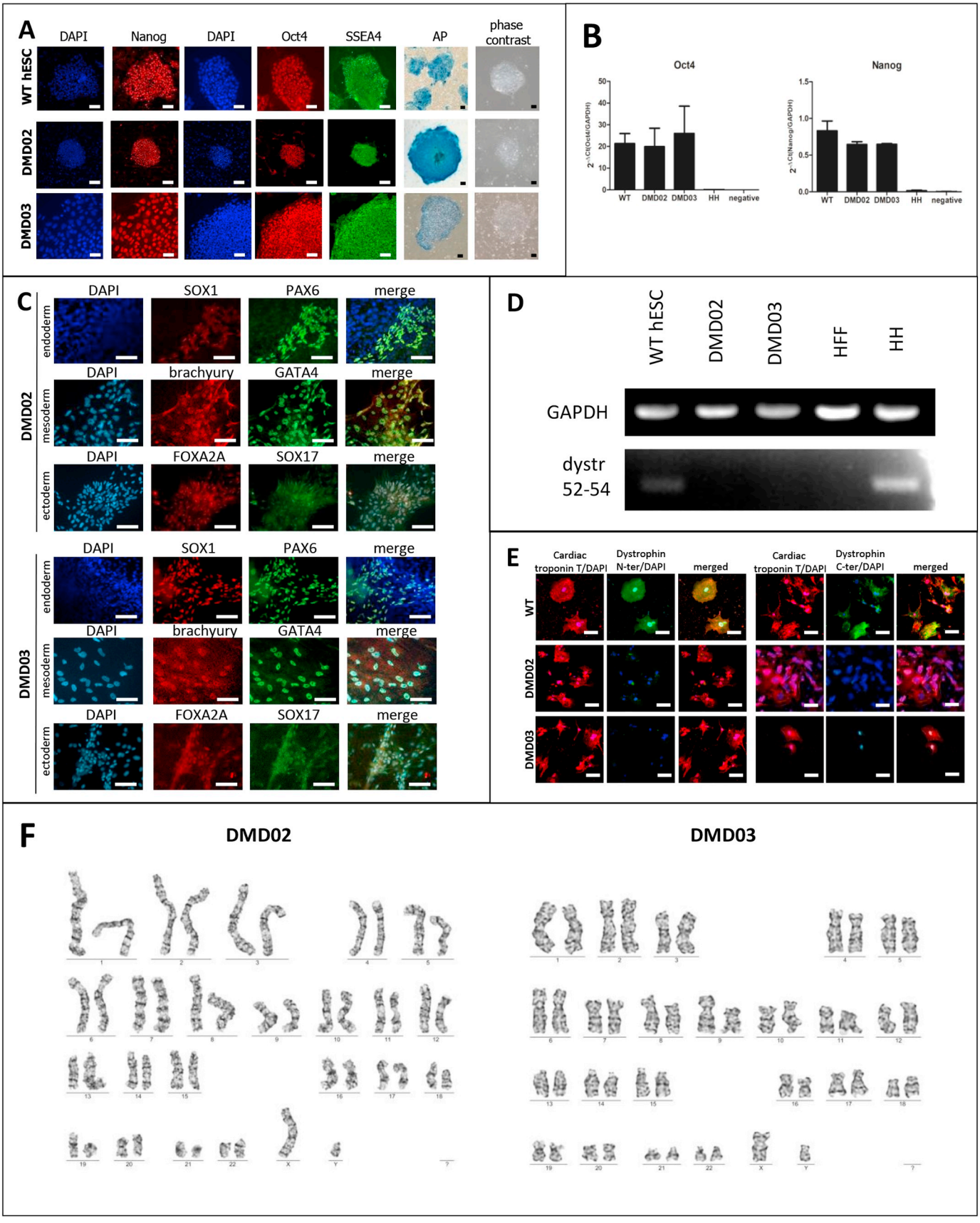
A
DAPI
Nanog
DAPI
Oct4
SSEA4
AP
phase contrast
WT hESC
DMD02
DMD03
B
Oct4
Nanog
WT
DMD02
DMD03
HH
negative
C
DAPI
SOX1
PAX6
merge
endoderm
DMD02
mesoderm
brachyury
GATA4
ectoderm
FOXA2A
SOX17
DMD03
D
WT hESC
DMD02
DMD03
HFF
HH
GAPDH
dystr 52-54
E
Cardiac troponin T/DAPI
Dystrophin N-ter/DAPI
merged
Dystrophin C-ter/DAPI
WT
DMD02
DMD03
F
DMD02
DMD03

Fig. 1.

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
MUNIi001-A	DMD02	Male	10	Caucasian	DMD	Duchenne muscular dystrophy
MUNIi003-A	DMD03	Male	14	Caucasian	DMD	Duchenne muscular dystrophy

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	normal	Fig. 1, panel A
Phenotype	Qualitative analysis (i.e. Immunocytochemistry)	Assess staining/expression of pluripotency markers: Oct4, Nanog, Sox2	Fig. 1 panel A
	Quantitative analysis (i.e. Immunocytochemistry counting, Flow cytometry, RT-qPCR)	Assess % of positive cells or transcripts for antigen & cell surface markers DMD03 Oct3/4: 98%, Nanog: 98%, SSEA-4: 99% DMD02: Oct3/4: 97%, Nanog: 98%, SSEA-4: 99%	Fig. 1, panel A, calculated from ICC images, Fig. 1 panel B as qPCR result
Genotype	Karyotype (G-banding) and resolution	Both 46XY, Resolution 450–500	Fig. 1 panel F
Identity	STR analysis	DNA Profiling performed with 17 sites, matched	submitted in archive with journal
Mutation analysis	MLPA	X-linked mutation on exons 45–50 (DMD02) /48–50 (DMD03)	Supplementary Table 1
Microbiology and virology	Mycoplasma	Mycoplasma testing by luminescence. Negative	Not shown, but available with authors
Differentiation potential	Directed differentiation	ectodermal markers (SOX1 and PAX6), endodermal markers (SOX17 and FOXA2A) and mesodermal markers (GATA4 and brachyury) expression showed by ICC, plus successful differentiation into CMs	Fig. 1 panel C and D
Donor screening	HIV 1 + 2 Hepatitis B, Hepatitis C	Negative	N/A
Genotype additional info	Blood group genotyping	N/A	N/A
	HLA tissue typing	HLA typed Class I and Class II	N/A

hiPSC lines and WT control, while no expression was found in differentiated human heart tissue (Fig. 2B). The ability of the generated DMD hiPSC lines to differentiate *in vitro* into all three germ layers was tested by incubation with ectodermal, endodermal or mesodermal inducing medium (Fig. 1C). We observed an expression of early differentiation markers of ectoderm (SOX1 in red, PAX6 in green), endoderm (SOX17 in green, FOXA2A in red) and mesoderm (GATA4 in green, brachyury in red) in both DMD hiPSC lines. Multiplex ligation-dependent probe amplification (MLPA) analysis showed the presence of the same mutation in the DMD hiPSC lines (patient 1: deleted exons 45–50, referred to as DMD02; and patient 2: deleted exons 48–50, referred to as DMD03; Supplementary Table 1) and short tandem repeats analysis confirmed their origin from the patient derived fibroblasts (data stored with the editors). The obtained DMD hiPSC lines presented no dystrophin mRNA expression in pluripotent state compared to the WT hiPSC (Fig. 1D, human heart sample used as positive control, human foreskin fibroblasts used as negative control). Dystrophin absence was confirmed in CMs differentiated from DMD hiPSCs by western blot (Jelinkova et al., 2019). All hiPSC derived CMs had positive signal for cardiac troponin T as CM marker (Caluori et al., 2019) (two antibodies recognizing N-terminus (N-ter) and C-terminus (C-ter) of dystrophin protein were used, Fig. 1E). All hiPSC derived CMs had positive signal for cardiac troponin T as CM marker. The generated hiPSC lines are karyotypically healthy (both 46, XY, Supplementary files).

Materials and methods

Tissue processing

The fibroblasts of two DMD patients were derived from skin/muscle (for DMD02/DMD03, respectively) biopsies. Informed consents approved by Ethics Committee (Faculty of Medicine, Masaryk University) were signed by parents of the patients beforehand. The biopsy tissues were cut into 0.5–1 mm^3 pieces, seeded onto 6 well plates (TPP) in medium containing KnockOut DMEM (Invitrogen), 10% heat-inactivated fetal bovine serum, 0,1 mM β-mercaptoethanol, 1% penicillin-streptomycin, 1% L-glutamine, 1% non-essential amino acids and layered with cover glasses. The tissue samples were kept in the incubator for 5 days without movement. The medium was further changed every 2–3 days and cells passaged at day 10 *in vitro* of cultivation using trypsin.

Pluripotent stem cell lines derivation and characterization

The hiPSC lines were obtained by reprogramming of cultivated human fibroblasts using CytoTuneTM iPS reprogramming kit (A13780–01; Life Technologies, Carlsbad, CA, USA) according to manufacturer's recommendations. DMD hiPSC lines are referred to as DMD02 (MUNIi001-A) and DMD03 (MUNIi003-A) and were further cultivated either on feeder layer of mouse embryonic fibroblasts in DMEM/F12, 15% KnockOut Serum replacement, 1% L-glutamine, 1% non-essential amino acids (all Invitrogen, Carlsbad, CA, USA), 0,1 mM β-mercaptoethanol (Sigma Aldrich, St.Louis, MO, USA) and 10 ng/ml FGF2 (Peprotech, Rocky Hill, NJ, USA)] or on Matrigel™ (Corning, New York, USA)-coated dish in feeder conditioned medium(Krutá et al., 2014). DMD hiPSCs were characterized using immunocytochemical staining of pluripotency markers (Nanog, Oct4, SSEA4, Alkaline phosphatase was tested using Blue Microwell Substrate kit AB0100 and AB0200, Sigma Aldrich) and compared with wild type (WT) human embryonic stem cell (hESC) line (Center for Cell Therapy line 14, CCTL14)(Krutá et al., 2014). The ability of the DMD hiPSC lines to differentiate *in vitro* into all three germ layers was tested by incubation for:

i) ectodermal induction: 2 days in RPMI1640, 1× B27, N2 supplement (Gibco, Thermo Fisher Scientific) plus SB431542 (Sigma Aldrich); 4 days in RPMI1640, 1× B27, N2 supplement
ii) endodermal induction: 6 days in RPMI1640 with B27, Activin A 50 ng/ml, BMP4 20 ng/ml, (both R&D)
iii) mesodermal induction: 3 days in RPMI1640 with B27, Activin A 10 ng/ml, BMP4 10 ng/ml; 1 day addition of IWP2 (Sigma Aldrich); 2 days in medium without IWP2.

Expression of early differentiation markers of ectoderm, endoderm and mesoderm were detected in both DMD hiPSC lines after 6 days of differentiation.

Immunocytochemistry

Cells were fixed with 2% paraformaldehyde for 15 min and ice-cold methanol for 5 min, then permeabilized with 0.5% Triton X-100 and blocked with 1% bovine serum albumin for 60 min. Samples were incubated overnight at 4 °C with appropriate primary antibodies and 60 min at room temperature with secondary antibodies. Nuclei were counterstained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI, Sigma Aldrich, St. Louis, MO, USA) and microscopic images were obtained using confocal microscope LSM700 and processed with Zen system (both Carl Zeiss, Oberkrochen, Germany).

Mutation validation

DMD hiPSCs lines were tested for the mutation presence by clinical MLPA analysis. MLPA was performed using SALSA MLPA P034 DMD mix 1 probemix and SALSA MLPA P035 DMD mix 2 probemix (LOT B1–1014, B1–0216, MRC Holland, Amsterdam, Netherlands), according to manufacturer's instructions. MLPA fragments were separated on ABI PRISM 3130 Genetic analyser (Applied Biosystems) and data were analysed using Coffalyser software (MRC Holland).

PCR

Total mRNA from cell culture and human heart samples was lysed using RNA Blue reagent (Top-Bio, Prague, Czech Republic) according to the manufacturer's instructions, and the total mRNA was isolated using the RNeasy Micro Kit (Qiagen, Hilden, Germany). mRNA concentration and purity were determined using NanoDrop (NanoDrop technologies, Wilmington, Germany). For reverse transcription PCR (rtPCR), cDNA was synthesized by Moloney Mouse Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA, USA) at 37 °C for 1 h followed by 5 min at 85 °C. The consequent semi quantitative PCR was performed using Taq polymerase (Top-Bio, T032, Prague Czech Republic) and included denaturation at 95 °C for 10 min followed by 35 cycles of 95 °C for 10 s, annealing at 64 °C for 45 s and extension at 72 °C for 20 s; the final extension step proceeded at 72 °C for 10 min. For quantitative PCR, LightCycler® 480 SYBR Green I Master kit was used according to manufacturer's instructions. Annealing at 60 °C and 45 cycles were used for the PCR reaction on LightCycler LC480 Instrument (both Roche, Basil, Switzerland). The PCR primers (Generi-Biotech, Hradec Kralove, Czech Republic) and annealing conditions are shown in Reagent Details Table. The PCR product was then run on 1% agarose gel for 100 min/130 V/500 mA and photos were obtained using UV lamp DNR MiniBis Pro (Bio-Imaging Systems, Neve Yamin, Israel).

Line identity validation

Cell culture identity was analysed using short tandem repeat analysis using ABI PRISM 3130 Genetic analyser and PowerPlex® ESI17 Fast System (Promega) according to manufacturer's recommendations (Tables 1 and 2).

Karyotype

Karyotypes were analysed in passages P83 (DMD02) and 59 (DMD03) (number indicates passage number on feeder layer) after incubation with colchicine (2 µg/ml) for 4 h and treatment with hypotonic solution consisting of cultivation medium and water in ratio 1:3. Chromosome banding was achieved using Giemsa staining. Chromosomes from 10 mitoses in each sample evaluated in resolution 400–450 bands. BX53 microscope (Olympus, Tokio, Japan) was used with 1250× magnification and photos were taken using ProgRes MF camera (Jenoptik, Jena, Germany). Images were evaluated using LUCIA Cytogenetics 2 Karyo (Laboratory Imaging s.r.o., Prague, Czech Republic).

Key resources table

Unique stem cell lines identifier	*MUNIi001-A* *MUNIi003-A*
Alternative names of stem cell lines	*DMD02* *DMD03*
Institution	Department of Biology, Faculty of Medicine, Masaryk University, Brno 625 00, Czech Republic;
Contact information of distributor	*Vladimír Rotrekl PhD,vrotrekl@med.muni.cz*
Type of cell lines	*hiPSC*
Origin	*human*
Cell Source	*Leg skin biopsy fibroblasts*
Clonality	*mixed*
Method of reprogramming	*Sendai virus*
Multiline rationale	*Mutation in DMD gene with deletion of exons 45–50 (MUNIi001-A) or exons 48–50 (MUNIi003-A)*
Gene modification	*Yes*
Type of modification	*Hereditary*
Associated disease	*Duchenne muscular dystrophy*
Gene/locus	*Xp21*
Method of modification	*N/A*
Name of transgene or resistance	*N/A*
Inducible/constitutive system	*N/A*
Date archived/stock date	*MUNIi003-A 12.4.2015* *MUNIi001-A 16.11.2015*
Cell line repository/bank	*https://hpscreg.eu/cell-line/MUNIi001-A* *https://hpscreg.eu/cell-line/MUNIi003-A*
Ethical approval	*Faculty of Medicine, Masaryk University Ethics Committee (Brno, Czech Republic), approval number 37/2011*

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101562.

Declaration of Competing Interest

The authors declare no conflict of interest.

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