



Published in final edited form as:

Stem Cell Res. 2018 May ; 29: 111–114. doi:10.1016/j.scr.2018.03.023.

Generation of Duchenne muscular dystrophy patient-specific induced pluripotent stem cell line lacking exons 45–50 of the dystrophin gene (IITi001-A)

Binyamin Eisen, MSc^{a,*}, Ronen Ben Jehuda, MSc^{a,b,*}, Ashley J. Cuttitta, MSc^c, Lucy N. Mekies, MSc^a, Irina Reiter, MSc^a, Sindhu Ramchandren, MD^d, Michael Arad, MD^{e,f}, Daniel E. Michele, PhD^{c,#}, and Ofer Binah, PhD^{a,#}

^aDepartment of Physiology, Biophysics and Systems Biology, Ruth and Bruce Rappaport Faculty of Medicine, Technion – Israel Institute of Technology, Haifa, Israel

^bDepartment of Biotechnology, Technion – Israel Institute of Technology, Haifa, Israel

^cDepartment of Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor, Michigan, USA

^dDepartment of Neurology, University of Michigan Medical School, Ann Arbor, MI, USA

^eLeviev Heart Center, Sheba Medical Center, Ramat Gan, Israel

^fSackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

Abstract

Duchenne muscular dystrophy (DMD) is an X-linked progressive muscle degenerative disease caused by mutations in the dystrophin gene. We generated induced pluripotent stem cells (iPSCs) from a 13-year-old male patient carrying a deletion mutation of exons 45–50; iPSCs were subsequently differentiated into cardiomyocytes. iPSCs exhibit expression of the pluripotent markers (SOX2, NANOG, OCT4), differentiation capacity into the three germ layers, normal karyotype, genetic identity to the skin biopsy dermal fibroblasts and the patient-specific dystrophin mutation.

Resource Table

Unique stem cell line identifier	IITi001-A
----------------------------------	-----------

Corresponding authors: Ofer Binah, PhD, Cardiac Research Laboratory, Department of Physiology, Biophysics and Systems Biology, Ruth and Bruce Rappaport Faculty of Medicine, Technion – Israel Institute of Technology, 1 Efron Street, POB 9649, Haifa 31096, Israel, binah@tx.technion.ac.il. Daniel Michele, PhD, Department of Molecular and Integrative Physiology, Department of Internal Medicine, University of Michigan, North Campus Research Complex, 2800 Plymouth Road, Bldg 26, Room 207S, Ann Arbor, MI 48109-2800, USA, dmichele@umich.edu.

*These authors contributed equally to this study.

#These authors contributed equally to this study.

Additional files:

STR analysis

Data is available with the authors.

Alternative name(s) of stem cell line	29.2
Institution	Technion – Israel Institute of Technology (IIT), Haifa, Israel. University of Michigan, Ann Arbor, Michigan, USA.
Contact information of distributor	Daniel Michele, PhD dmichele@umich.edu
Type of cell line	iPSC
Origin	Human
Additional origin info	Age: 13 Sex: male
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogramming	Sendai virus
Genetic Modification	NO
Type of Modification	N/A
Associated disease	Duchenne muscular dystrophy (DMD)
Gene/locus	Dystrophin del. Ex. 45–50
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	17/07/17
Cell line repository/bank	N/A
Ethical approval	Consent was obtained according to IRB HUM00030934 approved by the University of Michigan Human IRB Committee

Resource utility

This iPSC line was generated to study DMD disease pathophysiology and the electrophysiological abnormalities of iPSC-derived cardiomyocytes (iPSC-CMs) using methods such as: patch clamp, microelectrode array, calcium-contraction imaging.

Resource Details

Duchenne muscular dystrophy (DMD) is an X-linked muscle degenerative disease resulting in progressive weakness, loss of ambulation in the early teen years, and death by the end of the 3rd decade of life due to respiratory and cardiac failure¹. DMD is caused by mutations in the dystrophin gene, the longest gene in the human DNA²; loss of dystrophin expression leads to destabilization of the dystrophin glycoprotein complex (DGC), sarcolemma instability and fibrotic replacement of degenerated muscle³. To date, there are no curative treatments for DMD, and dilated cardiomyopathy (DCM) is a major cause of morbidity and mortality¹. The most common murine model for DMD is the *mdx* mouse¹; however, while DMD patients develop DCM, *mdx* mice develop extremely mild cardiomyopathic phenotypes³. Furthermore, *mdx* mice cardiomyocytes are unstable in primary culture preventing adaptation of this model for *in vitro* research. Therefore, the *mdx* model carries major limitations specifically regarding the cardiac pathology. To investigate the DMD cardiac manifestations using a cellular model, we generated human patient iPSCs that were

subsequently differentiated into cardiomyocytes. After informed consent form was obtained according to the Helsinki Committee at the University of Michigan, a skin biopsy was obtained from a 13-year-old patient carrying a deletion of exons 45–50 in the dystrophin gene. The mutation was first diagnosed by multiplex PCR amplification and a deletion of exons 45–50 was confirmed independently by MLPA and Southern blotting of exons 44 and 51². Human dermal fibroblasts were transfected with four Yamanaka's factors resulting in iPSCs exhibiting pluripotent stem cell-like morphology. To characterize the iPSCs, we performed immunofluorescence staining for the pluripotent markers SOX2, NANOG, OCT4 (Figure 1A). Furthermore, teratoma assay analysis demonstrated differentiation capacity to the three germ layers: ectoderm, represented by neuronal rosettes; mesoderm, represented by striated muscle; and endoderm, represented by epithelial lining (Figure 1B). Karyotype analysis revealed normal 46 XY chromosomes (Figure 1C). To verify that the iPSCs are genetically identical to the human dermal fibroblasts from which they were generated, short tandem repeat (STR) profiling of 24 loci was performed, and complete match was found (to protect the patient's identity, data is available with the authors). To investigate the phenotypes of dystrophin-mutated cardiomyocytes, iPSCs were differentiated into contracting cardiomyocytes using a directed differentiation protocol^{4, 5}. To verify that the deletion mutation of exons 45–50 was preserved through the process of reprogramming, Sanger sequencing was performed on a dystrophin RT-PCR product (spanning exons 44–51) generated from iPSC cardiomyocyte RNA (Figure 1D, red arrow indicates the deletion point). Furthermore, mycoplasma test was performed and the result was negative (Figure 1E).

Materials and Methods

Generation of induced Pluripotent Stem Cells (iPSCs) from the patient

iPSCs were generated from the patient's dermal fibroblasts using Sendai virus CytoTune-iPS 2.0 Sendai Reprogramming Kit, #A16517 (Thermo Fisher, Waltham, MA, USA) for the transfection of Yamanaka's 4 factors: OCT4, KLF4, c-Myc, SOX2. Subsequently, iPSCs were cultured on Matrigel (GFR, BD Biosciences, Franklin Lakes, NJ, USA) coated 6-well plates with mTeSR1 medium (Stemcell Technologies, Vancouver, Canada) and cultured in 37°C with 5% CO₂. Every 5 days, iPSCs were passaged in a ratio of 1:6 by dissociation using 1 ml/well Versene solution (Invitrogen, Life Technologies, Woburn, MA, USA) at 37°C for 7 minutes.

Karyotype analysis

Karyotype analysis was performed using standard G-banding chromosome analysis for 20 metaphase spreads by the Rambam Health Center cytogenetic laboratory according to standard procedures.

Genotyping

Extracted RNA was achieved using PureLink RNA mini kit (Thermo Fisher, Waltham, MA, USA). Subsequently, cDNA was produced using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, Waltham, MA, USA). PCR was performed (35 cycles; denaturation: 94° C for 30 sec, annealing: 61° C for 60 sec, elongation: 72° C for 45 sec;

Biometra 2005; Analytik Jena, Jena, Germany) to the *dystrophin* gene using the primers listed in Table 2 resulting in a product of 340 base pairs in length. Next, Sanger sequencing for the PCR product containing the mutation area within the DMD gene confirmed the deletion of exons 45–50.

Immunofluorescence staining

Immunofluorescence staining was performed according to standard procedures. Briefly, 3 days after iPSCs passaging, growth medium was discarded and iPSCs were washed with PBS (Biological Industries, Kibbutz Beit-Haemek, Israel). Subsequently, fixation was performed using 4% paraformaldehyde for 30 minutes and washed 3 times with PBS followed by permeabilization with 0.5% Triton X-100 in PBS for 15 minutes. Goat serum (#50-062Z, Invitrogen, Life Technologies, Woburn, MA, USA) was used as a blocking solution for 15 minutes, followed by incubation with primary antibody (as listed in Table 2) in antibody diluent (#ZUC025-100, Zytomed Systems, Berlin, Germany) overnight at 4° C. After 3 washes with PBS, secondary antibody was incubated for 1 hour at room temperature, followed by 3 washes and incubation with DAPI (1:500; Sigma Aldrich, St. Louis, MO, USA) for 5 minutes. Images were obtained using Zeiss Axio observer microscope (Zeiss, Oberkochen, Germany). SOX2-positive cells (500) were evaluated by cell-counting in immunofluorescence staining images.

Teratoma Formation

To evaluate the differentiation capacity of iPSCs *in vivo*, colonies from two 6-well plates were detached using 1 mg/ml type IV collagenase, washed 3 times in PBS and injected into the thigh muscle of severe combined immunodeficient (SCID) mice. Eight-to-12 weeks after injection, teratomas were observed, and images were obtained from formalin-fixed (4%) and paraffin-embedded teratoma sections stained with hematoxylin and eosin (H&E).

STR profiling

The analysis was performed using the Promega GenePrint 24 System in order to determine STR profile of 23 loci plus Amelogenin for gender determination (X or XY). In addition, the male-specific DYS391 locus is included to identify null Y allele results for Amelogenin. DNA sample from the kit (2800M Control DNA) was included in the analysis and served as positive control for the PCR step. The results were analyzed using the 3500xl Genetic Analyzer (Life Technologies, Carlsbad, CA, USA) and GeneMapper IDX software. Tested loci: AMEL, D3S1358, D1S1656, D2S441, D10S1248, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA, D21S11, D7S820, D5S818, TPOX, DYS391, D8S1179, D12S391, D19S433, FGA, D22S1045.

Differentiation into cardiomyocytes

iPSC-CMs were generated according to the directed differentiation by modulating Wnt/ β -catenin signaling as previously described⁵. Briefly, iPSCs were cultured on Matrigel (GFR, BD Biosciences, Franklin Lakes, NJ, USA) coated 6-well plates with mTeSR1 medium (Stemcell Technologies, Vancouver, Canada) for 5–6 days. To initiate differentiation, the cells were dissociated using 1 ml/well Versene solution (Invitrogen, Life Technologies,

Woburn, MA, USA) at 37°C for 7 minutes and reseeded on Matrigel coated plate at $8.5 \times 10^6/12$ well plate density in mTeSR1 medium supplemented with 5 $\mu\text{mol/l}$ ROCK inhibitor (Cayman Chemical, Ann Arbor, MI, USA). The medium was replaced daily, and after 2 days when the monolayer of cells reached 100% confluence, the medium was changed to RPMI supplemented with B27 minus insulin (Invitrogen, Life Technologies, Woburn, MA, USA) containing 10 $\mu\text{mol/l}$ CHIR99021; this day was labelled as day 1 of differentiation. On the next day, the medium was changed to RPMI supplemented with B27 minus insulin. On day 4, the medium was changed to RPMI supplemented with B27 minus insulin, containing 10 $\mu\text{mol/l}$ of IWP-4 or 2. On day 6, the medium was changed to RPMI supplemented with B27 minus insulin. Finally, from day 8 onwards, the medium was changed to RPMI supplemented with B27 complete supplement (Invitrogen, Life Technologies, Woburn, MA, USA).

Mycoplasma test

Supernatant was collected for mycoplasma test after 48 hours of iPSCs culture; the test was performed using the PCR-based EZ-PCR Mycoplasma Test Kit (Biological Industries, Kibbutz Beit-Haemek, Israel).

References

1. McNally EM, Kaltman JR, Benson DW, Canter CE, Cripe LH, Duan D, Finder JD, Groh WJ, Hoffman EP, Judge DP, Kertesz N, Kinnett K, Kirsch R, Metzger JM, Pearson GD, Rafael-Fortney JA, Raman SV, Spurney CF, Targum SL, Wagner KR, Markham LW. Working Group of the National Heart, Lung and BI, Parent Project Muscular Dystrophy. Contemporary cardiac issues in Duchenne muscular dystrophy. Working Group of the National Heart, Lung, and Blood Institute in collaboration with Parent Project Muscular Dystrophy. *Circulation*. 2015; 131:1590–8. [PubMed: 25940966]
2. Lalic T1, Vossen RH, Coffa J, Schouten JP, Guc-Scekic M, Radivojevic D, Djuricic M, Breuning MH, White SJ, den Dunnen JT. Deletion and duplication screening in the DMD gene using MLPA. *Eur J Hum Genet*. 2005; 13:1231–4. [PubMed: 16030524]
3. Khouzami L, Bourin M-C, Christov C, Damy T, Escoubet B, Caramelle P, Perier M, Wahbi K, Meune Christophe, Pavoine C, Pecker F. Hospitalier Henri Mondor-Albert Chenevier G. Delayed cardiomyopathy in dystrophin deficient *mdx* mice relies on intrinsic glutathione resource. *Am J Pathol*. 2010; 177:1356–1364. [PubMed: 20696779]
4. Ben Jehuda R, Eisen B, Shemer Y, Mekies LN, Szantai A, Reiter I, Cui H, Guan K, Haron-Khun S, Freimark D, Sperling SR, Gherghiceanu M, Arad M, Binah O. CRISPR correction of the PRKAG2 gene mutation in the patient's induced pluripotent stem cell-derived cardiomyocytes eliminates electrophysiological and structural abnormalities. *Hear Rhythm*. 2018; 15:267–276.
5. Lian X, Zhang J, Azarin SM, Zhu K, Hazeltine LB, Bao X, Hsiao C, Kamp TJ, Palecek SP. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/ β -catenin signaling under fully defined conditions. *Nat Protoc*. 2013; 8:162–175. [PubMed: 23257984]

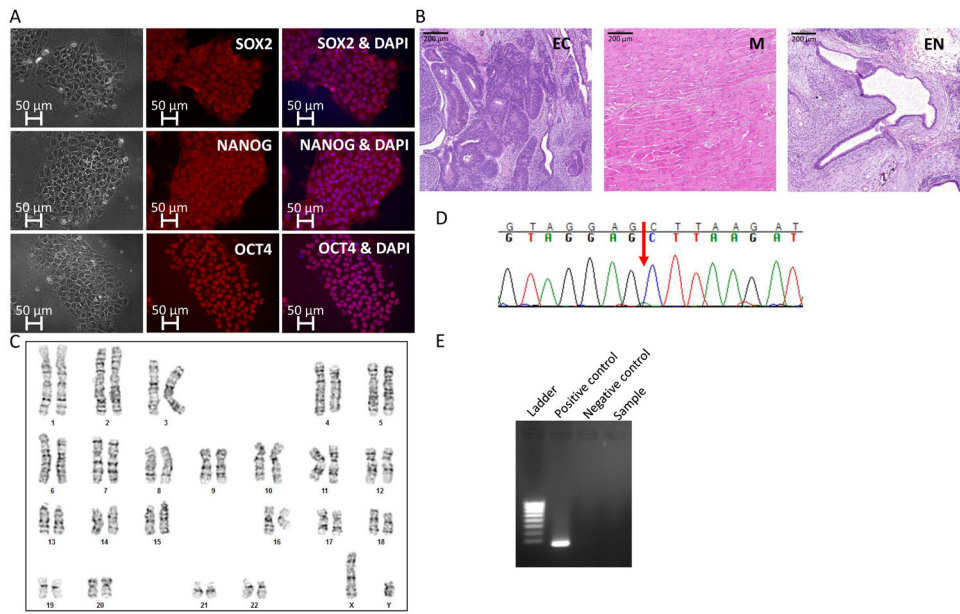


Figure 1.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

Characterization and validation

Classification	Test	Result	Data
Morphology	Photography	iPSCs exhibit pluripotent stem cell-like morphology	Figure 1A, left panel
Phenotype	Qualitative analysis (Immunofluorescent stainings)	Positive for pluripotency markers: OCT4, NANOG, SOX2	Figure 1A
	Quantitative analysis (Immunofluorescent counting)	97% SOX2-positive iPSCs	N/A
Genotype	Karyotype	46XY, G-banding Resolution 400	Figure 1C
Identity	STR analysis	Profile of 24 loci identifies complete match of the iPSCs and the human dermal fibroblasts (HDF) from which they were generated	Available with the authors
Mutation analysis (IF APPLICABLE)	Sequencing	Homozygous, deletion of exons 45–50	Figure 1D
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR, Negative	Figure 1E
Differentiation potential	Teratoma formation and Directed differentiation into cardiomyocytes	Teratoma assay analysis demonstrates differentiation capacity to the 3 germ layers (ectoderm, mesoderm, and endoderm). Directed differentiation into cardiomyocytes resulted in contracting iPSC-CMs	Figure 1B
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 2

Reagents details

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit anti-SOX2	1:100	Santa Cruz Biotechnology Cat# sc20088, RRID: AB_2255358
Pluripotency Markers	Rabbit anti-NANOG	1:100	PeptoTech Cat# 500-P236, RRID: AB_1268274
Pluripotency Markers	Rabbit anti-OCT4	1:200	Santa Cruz Biotechnology Cat# sc9081, RRID: AB_2167703
Secondary antibodies	Cy2 Donkey Anti-Rabbit IgG	1:100	Jackson ImmunoResearch Cat# 711-225-152, RRID: AB_2340612
Secondary antibodies	Alexa Fluor 555 Donkey Anti-Rabbit IgG	1:100	Life Technologies Cat# A31572, RRID: AB_162543
Primers			
	Target	Forward/Reverse primer (5'-3')	
Genotyping	Dystrophin exons 44–51	ATCTGTTGAGAAATGGCGGC/ GATGGTGGGTGACCTTGAGG	