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Lab resource: Stem Cell Line

Establishment of a Duchenne muscular dystrophy patient-derived induced pluripotent stem cell line carrying a deletion of exons 51–53 of the dystrophin gene (CCMi003-A)

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A B S T R A C T

Duchenne's muscular dystrophy (DMD) is a neuromuscular disorder affecting skeletal and cardiac muscle function, caused by mutations in the dystrophin (*DMD*) gene. Dermal fibroblasts, isolated from a DMD patient with a reported deletion of exons 51 to 53 in the DMD gene, were reprogrammed into induced pluripotent stem cells (iPSCs) by electroporation with episomal vectors containing the reprogramming factors: OCT4, SOX2, LIN28, KLF4, and L-MYC. The obtained iPSC line showed iPSC morphology, expression of pluripotency markers, possessed trilineage differentiation potential and was karyotypically normal.

Resource utility

This iPSC line will be helpful to the study of disease mechanisms underlying muscular dystrophy and to screen novel compounds with potential therapeutic effects.

Resource details

Duchenne Muscular dystrophy (DMD) is an X-linked neuromuscular disorder affecting skeletal and cardiac muscle function caused by mutations in the dystrophin (*DMD*) gene (D'Amario et al., 2018). Dystrophin is localized under the sarcolemma and is connected to the dystrophin-associated protein complex (DAPC) that normally anchors the cortical actin cytoskeleton and the plasma membrane to the extracellular matrix (ECM) (Constantin, 2014). *DMD* mutations cause deficiency in full-length dystrophin protein expression (427 kDa) which lead to a general disorganization of the DAPC. Myofibers lacking dystrophin and a disorganized DAPC are sensitive to mechanical damage and have deregulated signaling which activates cell death (Farini et al., 2019; Nanni et al., 2016).

Following institutional ethical committee approval and patient informed consent, dermal fibroblasts were isolated by explant culture of a skin biopsy obtained from a 10-year-old male with DMD. To protect privacy, no identifying patient information is included. Subsequent to the skin biopsy the patient lost ambulation at age 13 years. Cardiac monitoring revealed the development of left ventricular dysfunction at 18 years. The patient's electrocardiogram while normal at biopsy age but at last exam showed increased RS in V1 and deep narrow Q waves in left precordial leads. The ejection fraction (EF) was normal at time of biopsy but had decreased to 40% when last measured by echocardiography. The shortening fraction (SF) followed a similar pattern and was 20% when last determined.

Patient fibroblasts (FBS DMD3) were reprogramed into induced pluripotent stem cells (iPSCs) by electroporation with episomal vectors encoding human L-MYC, LIN28, SOX2, KLF4, OCT3/4 and cultured under feeder-free defined conditions. Following 25 days of reprograming iPSC colonies were selected for manual isolation and expanded to P5–10.

The clonal iPSC line described in this publication was named CCMi003-A, and entered iPSC characterization by evaluating: iPSC pluripotent cell morphology (Fig. 1A) and expression of pluripotency marker SSEA4 by immunocytochemistry (Fig. 1B). FACS analyzes

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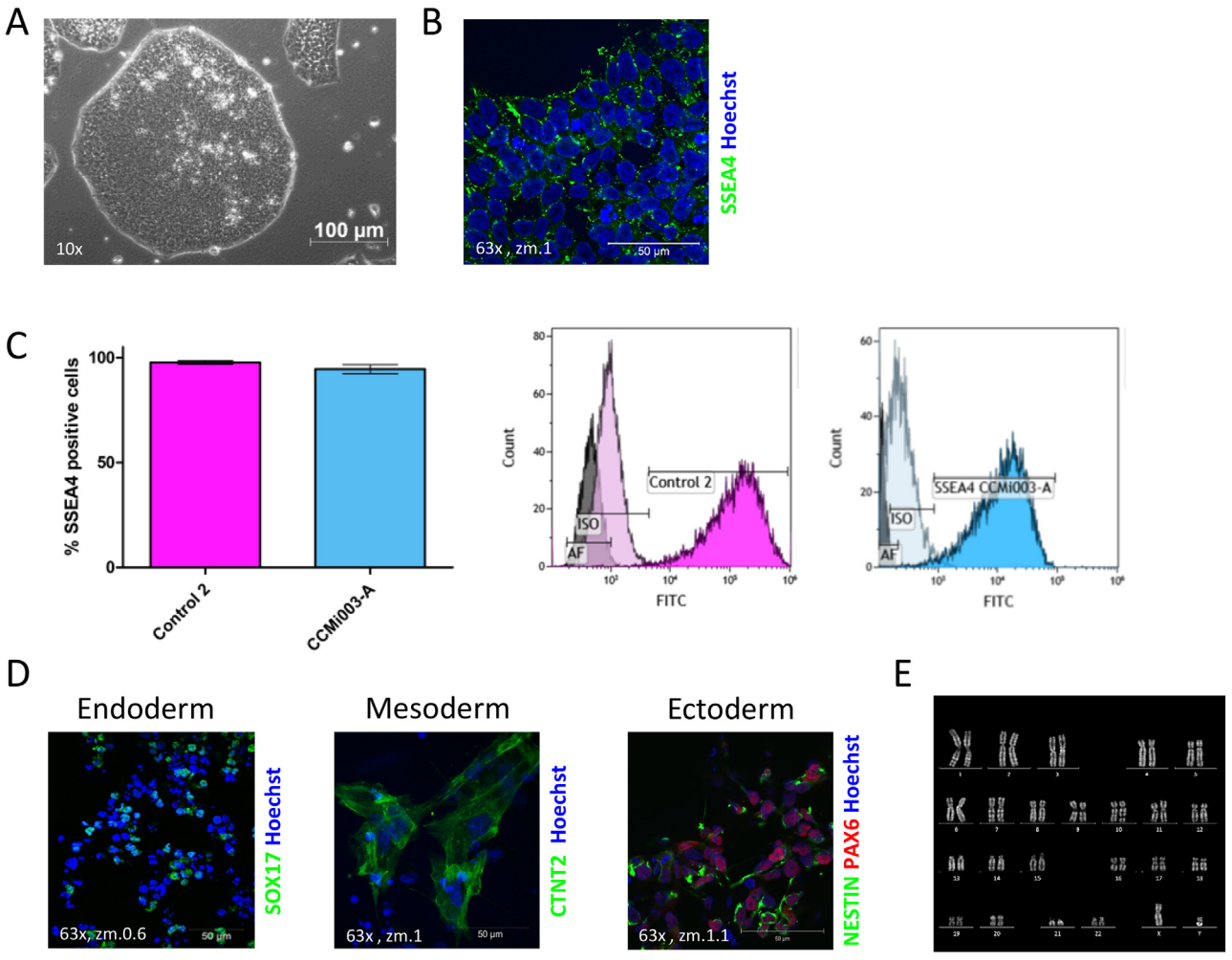

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Fig. 1. Derivation of the Duchenne's muscular dystrophy induced pluripotent stem cell line CCMi003-A. (A) Representative transmitted light image showing pluripotent cell morphology. (B) Expression of SSEA4 pluripotency-associated marker in CCMi003-A was determined by immunofluorescence. (C) FACS analysis showed large percentages of cells in CCMi003-A and a Control iPSC cell-line (Control 2) expressed the pluripotency marker SSEA4 as indicated by the summary data graph and the representative FACS analysis histograms (Control 2 v CCMi003-A, values are represented as mean ± SEM). (D) An in vitro trilineage differentiation assay revealed that CCMi003-A was capable of differentiation into all three germlayers. (E) Karyogram from CCMi003-A displaying a normal 46, XY karyotype with no measurable anomalies. (F) Multiplex ligation-dependent probe amplification assay (MLPA) revealed a normal genotype for Control iPSCs (lower electropherograms) while CCMi003-A presented deletions of DMD exons from 45 to 55 (upper electropherograms).

showed that 95% of CCMi003-A iPSCs were SSEA4 positive, with no significant differences compared to iPSC lines obtained from a healthy donor used as a characterization control (Fig. 1C). CCMi003-A had the capacity to differentiate into all three germ layers *in vitro* as indicated by immunofluorescence which showed expression of the mesodermal marker cardiac troponin T type 2 (CTNT2), the ectodermal markers NESTIN/PAX6 and the endodermal marker SOX17 (Fig. 1D). Karyotype analyzes, performed on 30 metaphases showed that CCMi003-A has a normal 46,XY karyotype (Fig. 1E). Short Tandem Repeat (STR) analyzes of 17 total loci (LGC Standards and ATCC) showed that CCMi003-A iPSCs were unique (no match to any other cell line in the ATCC-STR database or DSMZ) and matched to the original source fibroblasts (FBS DMD3).

Multiple ligation probe amplification (MLPA) assay performed on DNA extracted from CCMi003-A revealed the presence of a deletion of *DMD* gene exons 51, 52, 53 (Fig. 1F). CCMi003-A tested negative for mycoplasma (see supplementary materials). Mycoplasma-free CCMi003-A iPSCs were harvested and frozen in mFreSR™ (Stemcell Technologies) and transferred to long-term nitrogen storage.

Materials and methods

Reprograming of patient's fibroblasts to iPSCs

The patient's fibroblasts were isolated from a skin biopsy by explant culture. Fibroblasts were transfected with 1.25 μg of episomal vectors (pCXLE-hUL, pCXLE-hSK, pCXLE-hOCT3/4-shp53-F; Addgene) by electroporation (1650 V, 10 s, 3 pulse; Neon™ transfection system, Invitrogen), transferred into a single well of a 6 well plate pre-coated with human recombinant vitronectin (Life Technologies) and cultured at 37 °C with 5% CO_2. On day 3 post transfection transfected fibroblasts were transferred to a reprograming media (ReproTeSR™, Stemcell Technologies) which was replaced every day. Suitable individual colonies were harvested when they reached 1000 μm in diameter by manual isolation using a 25 gauge sterile syringe and transferred into an individual well of a 12-well plate containing mTeSR1™ media (Stemcell Technologies) supplemented with RevitaCell™ (Life Technologies). iPSCs were maintained in mTeSR1™ media with daily media changes. At 80–90% confluency, iPSCs were non-enzymatically passaged with ReLeSR™ (Stemcell Technologies) every 3–4 days and replated as small aggregates in mTeSR1™ media containing RevitaCell™. Stock vials of iPSCs were harvested in mFreSR™ (Stemcell Technologies) and stored at -180 °C for future experiments (Table 1).

Pluripotency marker immunofluorescence

CCMi003-A were cultured in vitronectin-coated chamber slides and fixed in 4% formaldehyde (10 min RT). Celle were treated with 0.1% Triton-X 100 in PBS for 5 min for permeabilization and with 3% BSA in PBS for 1 h for blocking. Antibodies were diluted in 0.3% BSA in PBS and incubated at 4 °C overnight (primary) and 1 h at RT (secondary). Nuclei were counterstained with Hoechst 33342 (Invitrogen; 1:500 in PBS) for 15 min at RT. iPSCs were analyzed with a confocal microscope (LSM710, Zeiss and ZEN 2010 D analysis software). All antibody details are listed in Table 2.

Flow cytometry

iPSCs were detached using ReLeSR™ (Stemcell Technologies), resuspended in PBS containing 0.1% BSA and 0.5 mM EDTA, fixed for 20 min on ice with BD Cytofix™ buffer (BD Biosciences) and stained with SSEA4 antibody (1 h, 4 °C) followed by the specific fluorescently tagged secondary antibody (1 h 4 °C). Antibodies were diluted in 0.1% BSA, 0.5 mM EDTA in PBS solution. Cells were analyzed using the Gallios (Beckman Coulter) or FACSCalibur (BD Biosciences) flow cytometers. Data was analyzed with Kaluza software (version 2.5, Beckman Coulter). An iPSC line from a healthy donor (Con2) was used as a characterization control.

In vitro trilineage differentiation potential

iPSCs were differentiated along ectodermal or endodermal lineages using the STEMdiff™ trilineage differentiation kit (Stemcell Technologies) according to the manufacturer's instructions. For differentiation along the mesodermal lineage, iPSCs were treated with small molecules targeting the Wnt pathway in order to induce cardiomyocyte differentiation according to Lian et al. (2013). Differentiated cells were stained with the specific antibodies recognising relevant lineage markers: ectoderm, XY; endoderm, SOX17; mesoderm, cardiac troponin type 2 (CTNT2); ectoderm, (NESTIN and PAX6). All antibody details are listed in Table 2. Nuclei were counterstained with Hoechst 33342 (Invitrogen; 1:500 in PBS) for 15 min at RT. Differentiated cells were analyzed by confocal microscopy (LSM710, Zeiss and ZEN 2010 D analysis software).

Karyotyping

Metaphase chromosomes were prepared from iPSC cultures at passage 30 (P30). After 48–96 h, colcemid (10 μg/ml) was added for 3 h at 37 °C. Cells were incubated in hypotonic solution (sodium citrate 0.6%, KCl 0.13%) at RT for 10 min, washed with Ibraimov solution (acetic acid 5%), fixed in Optichrome (28 °C, 42% rH) with methanol/acetic acid (3:1), Q-banded and photographed. Karyotype images were obtained at 100× magnification (Olympus BX microscope, U-CMAD3 Olympus camera). 30 metaphases were analyzed and karyotyped using an automated cytogenetic imaging system (MetaSystems Gmbh, Germany).

Dystrophin mutation analyses by multiple ligation probe amplification (MLPA)

DNA was extracted from iPSCs using the QIAamp DNA Mini Kit (Qiagen) according to manufacturer's instructions. The MLPA reaction was performed with the SALSA MLPA probe sets P034 and P035 (MRC Holland), following the manufacturer's instructions. The amplified products were run on the ABI PRISM 3130 genetic analyzer (Applied Biosystems) and the obtained data were analyzed using Coffalyser software (MRC Holland). DNA extracted from iPSCs obtained from a healthy individual's dermal fibroblast-derived iPSCs were used as controls and included in the run.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1A
Phenotype	Qualitative analysis: Immunocytochemistry	Positive for SSEA4	Fig. 1B
	Quantitative analysis: Flow cytometry	Positive for SSEA4 (95%)	Fig. 1C
Genotype	Karyotype (Q-banding) and resolution	46,XY, Resolution 400 band level	Fig. 1E
Identity	Microsatellite PCR (mPCR) OR	Not performed	
	STR analysis	18/18 loci matched	Available with the authors
Mutation analysis (IF APPLICABLE)	Sequencing	Not performed	
	MLPA analyses	Detection of deleted exons 51, 52 and 53 of the Dystrophin gene.	Fig. 1F
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR; Negative	Supplementary file 1 (S1)
Differentiation potential	Directed differentiation	Positive for: NESTIN/PAX6, ectoderm; CTNT2, mesoderm; SOX17, endoderm.	Fig. 1D
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	
	HLA tissue typing	Not performed	

STR analyzes

STR analyzes was performed by ATCC cell line authentication service. Seventeen STR loci plus the gender determining locus, Amelogenin, were amplified using the commercially available PowerPlex® 18D Kit from Promega. The cell line sample was processed using the ABI Prism® 3500xl Genetic Analyzer. Data were analyzed using GeneMapper® ID-X v1.2 software (Applied Biosystems). Appropriate positive and negative controls were run and confirmed for each sample submitted.

Mycoplasma analyses

To test the presence of Mycoplasma we used EZ-PCR Mycoplasma Detection Kit (Biological Industries) according to the manufacturer's instructions. A positive control was included in the kit.

Key resources table

Unique stem cell line identifier	CCMi003-A
Alternative name(s) of stem cell line	DMD3 C2
Institution	Centro Cardiologico Monzino-IRCCS, Milan, Italy.
Contact information of distributor	Aoife Gowran; agowran@ccfm.it
Type of cell line	iPSC
Origin	Human
Additional origin info	Age:10 (at skin biopsy) Sex: M Ethnicity if known: Caucasian
Cell Source	Dermal fibroblasts
Clonality	Clonal
Method of reprogramming	Episomal vectors containing: hL-MYC, hLIN28, hSOX2, hKLF4, hOCT4.
Genetic modification	YES
Type of modification	Congenital
Associated disease	Duchenne muscular dystrophy
Gene/locus	DMD gene, Xp21.2-p21.1
Method of modification	No modification
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	April 2019
Cell line repository/bank	The Telethon Biobank and the Eurobiobank
Ethical approval	The study was approved by the ethical committee of the European Institute of Oncology and Monzino Heart Centre (Istituto Europeo di Oncologia e dal Centro Cardiologico Monzino, IEO-CCM, CEA20150411, ammed. 20,190,528 AN/sd). Informed consent was given to donate biopsy material for use in research to The Telethon Biobank or The Eurobiobank which were accessed via grant numbers GTB12001 and GUP13013 respectively.

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Mouse anti-SSEA4	*1:200 (1:100 for FACS)*	*Abcam Cat# ab16287, RRID:AB_778073;*
Differentiation Markers	Rabbit anti-SOX17	*1:300*	*Cell Signaling Inc. Cat# 81778, RRID:AB_2650582;*
	Mouse anti-CTNT2	*1:300*	*Thermo Fisher Scientific Cat# MA-512960, RRID: AB_11000742;*
	Mouse anti-NESTIN	*1:150*	*Abcam Cat# ab22035, RRID: AB_446723*
	Rabbit anti-PAX6	*1:300*	*BioLegend Cat# PRB−278P, RRID: AB_291612*
Secondary antibodies	Anti-Mouse IgG, Alexa®Fluor 488	*1:400 (for SSEA4)*	*Thermo Fisher Scientific Cat# A11059, RRID:AB_2534106;*
	Anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody, Alexa®Fluor 633	*1:300 (for NESTIN)*	*Thermo Fisher Scientific Cat# A-21126, RRID: AB_2535768;*
	Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa®Fluor 594	*1:200 (for CTNT2)* *1:300 (for PAX6)*	*Thermo Fisher Scientific Cat# A-11037 RRID: AB_2534095*

Declaration of Competing Interest

The authors declare that they are unaware of any conflict of interests associated with this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101544.

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