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Lab Resource: Stem Cell Line

Derivation of the Duchenne muscular dystrophy patient-derived induced pluripotent stem cell line lacking *DMD* exons 49 and 50 (CCMi001DMD-A-3, Δ49, Δ50)

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A B S T R A C T

Duchenne muscular dystrophy (DMD) is caused by abnormalities in the dystrophin gene and is clinically characterised by childhood muscle degeneration and cardiomyopathy. We produced an induced pluripotent stem cell line from a DMD patient's dermal fibroblasts by electroporation with episomal vectors containing: hL-MYC, hLIN28, hSOX2, hKLF4, hOCT3/4. The resultant DMD iPSC line (CCMi001DMD-A-3) displayed iPSC morphology, expressed pluripotency markers, possessed trilineage differentiation potential and was karyotypically normal. MLPA analyses performed on DNA extracted from CCMi001DMD-A-3 showed a deletion of exons 49 and 50 (CCMi001DMD-A-3, Δ49, Δ50).

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Resource Table

Unique stem cell line identifier	CCMi001DMD-A-3, Δ49, Δ50
Alternative name(s) of stem cell line	DMD1c3
Institution	Centro Cardiologico Monzino-IRCCS
Contact information of distributor	Aoife Gowran, agowran@ccfm.it
Type of cell line	iPSC
Origin	Human
Additional origin info	Applicable for human ESC or iPSC Age: 34 Sex: M Ethnicity if known: Caucasian
Cell source	Dermal fibroblasts
Method of reprogramming	Episomal vectors containing the reprogramming factors: hL-MYC, hLIN28, hSOX2, hKLF4, hOCT3/4
Associated disease	Duchenne muscular dystrophy
Gene/locus	DMD gene, Xp21.2-p21.1
Method of modification	No modification
Gene correction	No
Name of transgene or resistance	NA
Inducible/constitutive system	NA
Date archived/stock date	24/07/17
Cell line repository/bank	
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; 29/01/2013-v.1d.28/11/2012). Skin biopsies were obtained from all patients after informed consent was given

1. Resource utility

This tool is valuable for the identification of novel DMD pathophysiological mechanisms downstream of dystrophin mutations and for screening putative therapies.

2. Resource details

Duchenne muscular dystrophy (DMD) is a severe early onset form of muscular dystrophy (MD) caused by either spontaneous mutations or

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inherited nonsense point mutations in the dystrophin gene (Flanigan 2012). Mutations in the dystrophin gene result in the failed expression of full-length dystrophin protein. Dystrophin deficiency is associated with skeletal muscle damage and impaired regeneration, and cardiomyopathy. Following institutional ethical committee approval and patient informed consent, dermal fibroblasts were isolated by explant culture of a skin biopsy obtained from a 34-year-old male with DMD. To protect privacy, no identifying patient information is included in this publication. The patient presented with an incidental finding of hyperkalaemia at 18 months of age. Loss of ambulation occurred at 10 years of age. The explanted patient fibroblasts were reprogrammed into induced pluripotent stem cells (iPSCs) by electroporation with plasmids encoding human L-MYC, LIN28, SOX2, KLF4, OCT3/4 (Okita et al. 2007) and cultured under feeder-free defined conditions. After 31 days of reprogramming, iPSC colonies were manually selected and culture expanded. The iPSC line described in this publication was named CCMi001DMD-A-3, and entered iPSC characterisation by evaluating: distinctive iPSC-like morphology and expression of the pluripotency markers by immunocytochemistry and FACS analyses (SSEA4, Sox2, Nanog and Oct4, Fig. 1A, 84% SSEA4$^+$ cells Fig. 1B); potential to differentiate along ectodermal, mesodermal

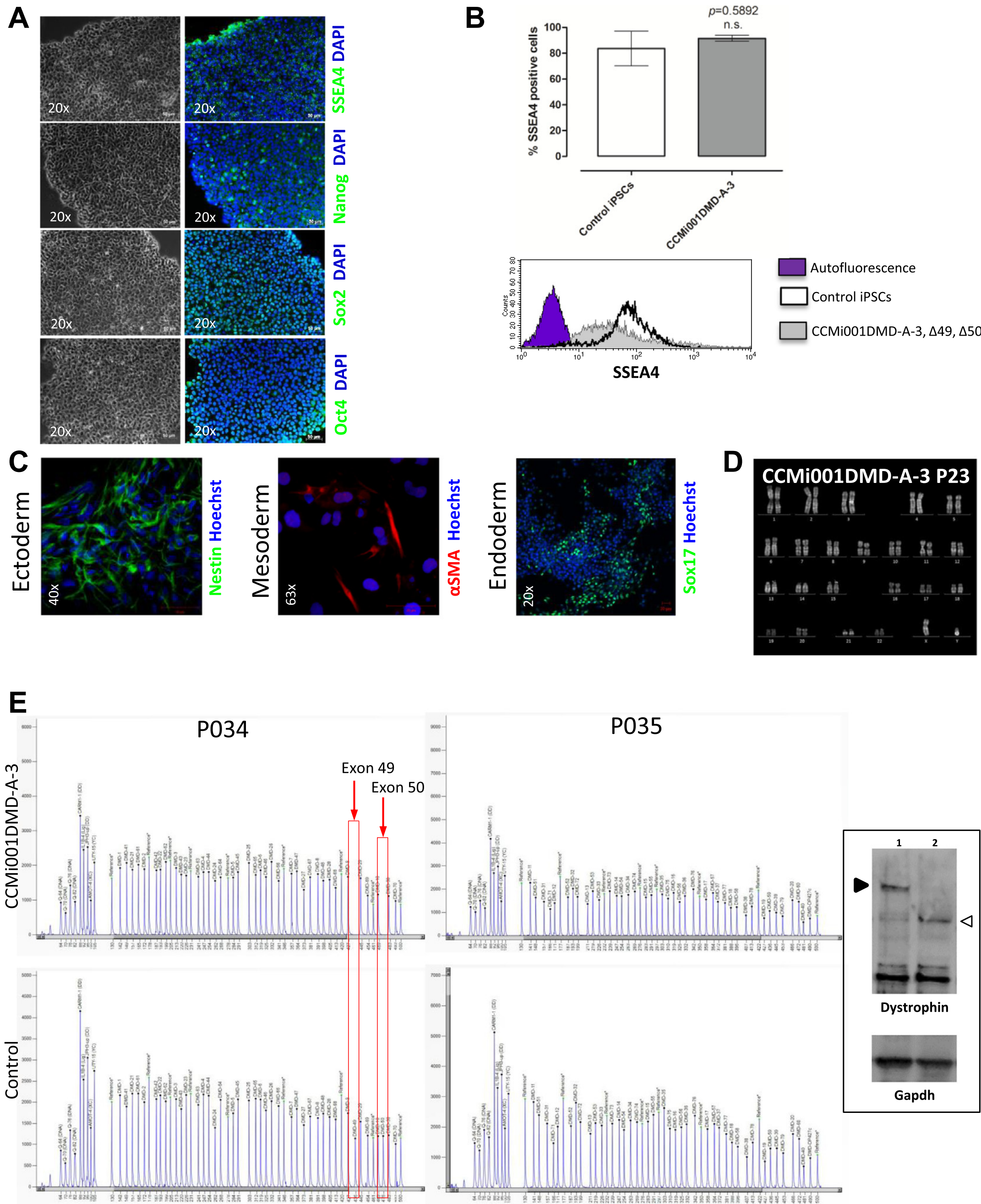

Fig. 1. Derivation of the Duchenne muscular dystrophy induced pluripotent stem cell line CCMi001DMD-A-3, Δ49, Δ50. **A** Expression of several pluripotency-associated markers in CCMi001DMD-A-3, Δ49, Δ50 was confirmed by immunofluorescence staining. **B** FACS analysis revealed that large percentages of cells in CCMi001DMD-A-3, Δ49, Δ50 and a Control iPSC line expressed the pluripotency marker SSEA4 as indicated above by the summary data histogram and a representative FACS analysis histogram (Control v CCMi001DMD-A-3, Δ49, Δ50, values are mean ± SEM, Student's *t* test, $n = 3$). **C** An *in vitro* trilineage differentiation assay revealed that CCMi001DMD-A-3, Δ49, Δ50 was capable of differentiation into all three germ layers. **D** Representative karyogram from CCMi001DMD-A-3, Δ49, Δ50 showing a normal 46, XY karyotype without any measurable anomalies. **E** Multiplex ligation-dependent probe amplification assay (MLPA) revealed a normal *DMD* genotype of Control iPSCs (lower electropherograms) with normal full-length dystrophin protein expression in cardiomyocytes derived from Control iPSCs (filled arrow, lane 1, inset); CCMi001DMD-A-3, Δ49, Δ50 contained deletions of DMD exons 49 and 50 (upper right electropherogram) this out-of-frame *DMD* mutation caused a lack of the full-length dystrophin protein expression (open arrow, lane 2, inset) in cardiomyocytes derived from CCMi001DMD-A-3, Δ49, Δ50.

and endodermal lineages (Nestin, αSMA and Sox17, Fig. 1C). Cytogenetic analysis was conducted on 30 mitosis from two independent cultures. The CCMi001DMD-A-3, iPSC line was karyotypically normal (size, shape, and number of chromosomes) at P23 (Fig. 1D). To detect the specific dystrophin mutation present in the CCMi001DMD-A-3 iPSC line, multiple ligation probe amplification (MLPA) analyses was performed on DNA extracted from CCMi001DMD-A-3 and a control iPSC line (derived from a healthy control subject's dermal fibroblasts). CCMi001DMD-A-3, showed a deletion of exons 49 and 50 (CCMi001DMD-A-3, Δ49, Δ50; Fig. 1E). Disruption of the *DMD* reading frame was confirmed by measuring the expression of dystrophin protein in functional CMs derived from CCMi001DMD-A-3, Δ49, Δ50 (DMD-A-3-CMs) by western blot and comparing it to CMs derived from iPSCs obtained from a healthy control subject's dermal fibroblasts (Control-CMs; Fig. 1E inset). DMD-A-3-CMs did not display full-length dystrophin protein expression (Fig. 1E inset, lane 2) which was observed at 427 kDa in Control-CMs (Fig. 1E inset, lane 1).

3. Materials and methods

3.1. Reprogramming of DMD patient's fibroblasts to iPSCs

All investigations were conducted according to the principles stated in the Declaration of Helsinki. Following informed consent under the regulations of the local ethics committee (European Institute of Oncology and Centro Cardiologico Monzino, Italy) the patient's fibroblasts were isolated from a skin biopsy by explant culture. Fibroblasts were transfected with episomal vectors (pCXLE-hUL, pCXLE-hSK, pCXLE-hOCT3/4; Addgene) by electroporation (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 fibroblast media was replaced with reprogramming media (ReproTeSR™, Stemcell Technologies) which was changed every day. 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 (every 3–4 days) with ReLeSR™ (Stemcell Technologies) 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).

3.2. Pluripotency marker immunocytochemistry

CCMi001DMD-A-3, Δ49, Δ50 iPSCs were cultured until confluent, split and transferred to vitronectin-coated chamber slides for analysis of pluripotency proteins. iPSCs were analysed with a confocal microscope (LSM710, Zeiss). All antibody details are listed in Table 2.

3.3. Flow cytometry

CCMi001DMD-A-3, Δ49, Δ50 were dissociated using Gentle Cell Dissociation Reagent (Stemcell Technologies). Non-specific staining was blocked using 5% Bovine Serum Albumin (BSA; Sigma-Aldrich) in PBS (Lonza). Cells were stained with a SSEA4 (1:100, 1 h; ab16287 Abcam) per reaction followed by goat anti-mouse IgM-FITC (1:200, 1 h; A11659 Life Technologies). Cells were analysed using a FACSCalibur™ flow cytometer (BD Biosciences).

3.4. In vitro trilineage differentiation potential assay

CCMi001DMD-A-3, Δ49, Δ50 iPSCs were subjected to monolayer differentiation using the STEMdiff™ trilineage differentiation kit (Stemcell Technologies) according to the manufacturer's instructions. On day 5 mesodermal- and endodermal-induced cells were fixed (4% formaldehyde, 3 min at RT) and permeabilised (0.5% triton, 3 min at RT). Unspecific binding was blocked in 3% BSA (2 h RT) and expression of αSMA and Sox17 were evaluated. Following 7 days of ectodermal-induction, cells were fixed, permeabilized and blocked as before and expression of Nestin was evaluated. Differentiated cells were analysed by confocal microscopy (LSM710, Zeiss). All antibody details are listed in Table 2.

3.5. Karyotyping

Metaphase chromosomes were prepared from CCMi001DMD-A-3, Δ49, Δ50 cultures at passage 23 (P23). After 48–96 h colcemid (10 μg/ml) was added for 3 h at 37 °C. iPSCs 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

Table 1
CCMi001DMD-A-3, Δ49, Δ50 summary information and, chracterisation and quality control analyses.

Classification	Test	Result	Data
Morphology	Photography	Created a visual record of normal pluripotent-like morphology: cobblestone pattern, refractive colony edges, prominent nucleoli	Fig. 1 A left hand panel
Phenotype	Immunocytochemistry	Assessed staining/expression of pluripotency markers: SSEA4, Nanog, Sox2, Oct3/4	Fig. 1 A right hand panel
	Flow cytometry	Determined cell surface expression of SSEA4 ($84\%^+$)	Fig. 1 B
Genotype	Karyotype (Q-banding) and resolution	e.g. 46XY, Resolution: 400 band level	Fig. 1 D
Identity	Microsatellite PCR (mPCR)	Not performed	–
	STR analysis	e.g. 17 STR loci plus gender determining locus and matched against the ATCC STR database CCMi001DMD-A-3, Δ49, Δ50 iPSCs CCMi001DMD original dermal fibroblasts	Fig. S1 Fig. S2
Mutation analysis	Sequencing	Not performed	–
	MLPA assay	Deletion of exons 49 and 50 from the DMD gene	Fig. 1E
	Western blot	Confirmed loss of dystrophin protein expression in iPSC derived cells that express dystrophin i.e. cardiomyocytes	Fig. 1E, inset
Microbiology and virology	Mycoplasma	Mycoplasma testing by RT-PCR, negative	Fig. S3
Differentiation potential	Directed differentiation	Determined the expression of markers for each of the three germlayers: Nestin, ectoderm; αSMA, mesoderm; Sox17, endoderm	Fig. 1 C
Donor screening (OPTIONAL)	Not performed	–	–
Genotype additional info (OPTIONAL)	Not performed	–	–
	Not performed	–	–

Table 2
RRID Requirement for antibodies: use http://antibodyregistry.org/ to retrieve RRID for antibodies and include ID in table as shown in examples.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat# and RRID
Pluripotency markers	Mouse anti-SSEA4	1:200 (for SSEA4 IF)	Abcam Cat# ab16287, RRID:AB_778073;
	Rabbit anti-Nanog	1:100 (for FACS)	Abcam Cat# ab21624, RRID:AB_446437;
	Rabbit anti-Sox2	1:100	Abcam Cat# ab97959, RRID:AB_2341193;
	Rabbit anti-Oct4	1:200	Abcam Cat# ab19857, RRID:AB_445175
Differentiation markers	Mouse anti-Nestin	1:300	Stemcell Technologies Cat# 60091AD, RRID:AB_2650581;
	Rabbit anti-Sox17	1:300	Cell Signaling Inc. Cat# 81778, RRID:AB_2650582;
	Mouse anti-αSMA	1:100	Millipore Cat# CBL171, RRID:AB_2223166
Western immunoblot	Rabbit anti-Dystrophin	1:500	Abcam Cat# ab15277, RRID:AB_301813;
	Mouse anti-Gapdh	1:2000	Santa Cruz Technologies Cat# sc-25778, RRID:AB_10167668
Secondary antibodies	Anti-Mouse IgG, Alexa®Fluor 488	1:400 (for SSEA4 IF) 1:200 (for SSEA4 FACS)	Thermo Fisher Scientific Cat# A11059, RRID:AB_2534106
	Anti-Rabbit IgG, Alexa®Fluor 488	1:200 (for Nanog) 1:400 (for Sox2/Oct4) 1:500 (for Sox17)	Thermo Fisher Scientific Cat# A11034, RRID:AB_2576217
	Anti-Mouse IgG2a Cross-Adsorbed Secondary Antibody, Alexa®Fluor 633	1:400 (for αSMA)	Thermo Fisher Scientific Cat# A-21136, RRID:AB_2535775
	Anti-rabbit IgG, HRP	1:1000	GE Healthcare Cat# NA 9340V, RRID:AB_772206
	Anti-Mouse IgG, HRP	1:10,000	GE Healthcare Cat# NA9310-1ml, RRID:AB_772193

microscope, U-CMAD3 Olympus camera). Metaphases (30) at approximately the 400 band level were analysed and karyotyped using an automated cytogenetic imaging system (MetaSystems Gmbh, Germany).

3.6. Dystrophin mutation analyses by multiple ligation probe amplification (MLPA)

Genomic DNA was extracted from iPSCs using the QIAamp DNA Mini Kit (Qiagen) according to manufacturer's instructions. The MLPA reaction was performed to detect all 79 exons of dystrophin gene using 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 analyser (Applied Biosystems) and the obtained data were analysed 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.

3.7. Dystrophin expression in iPSC cardiomyocytes

Differentiation of CCMi001DMD-A-3, Δ49, Δ50 along the cardiomyogenic lineage was performed following a monolayer small molecule-based directed differentiation protocol (Lian et al. 2013). Following 7 days of sustained beating (differentiation day 16), cardiomyocytes were processed for the determination of dystrophin and gapdh protein expression by western immunoblot analysis. All antibody details are listed in Table 2.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2017.10.018.

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