

Lab Resource: Stem Cell Line

Generation of induced pluripotent stem cells from a Becker muscular dystrophy patient carrying a deletion of exons 45–55 of the dystrophin gene (CCMi002BMD-A-9 Δ 45–55)



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ARTICLE INFO

Article history:

Received 4 April 2017

Received in revised form 9 January 2018

Accepted 18 January 2018

Available online 2 February 2018

ABSTRACT

Becker muscular dystrophy (BMD) is a dystrophinopathy caused by mutations in the dystrophin gene on chromosome Xp21. BMD mutations result in truncated semi-functional dystrophin isoforms. Consequently, less severe clinical symptoms become apparent later in life compared to Duchenne muscular dystrophy. Dermal fibroblasts from a BMD patient were electroporated with episomal plasmids containing reprogramming factors to create the induced pluripotent stem cell line: CCMi002BMD-A-9 that showed pluripotent markers, were karyotypically normal and capable of trilineage differentiation. MLPA analyses performed on DNA extracted from CCMi002BMD-A-9 showed an in-frame deletion of exons 45 to 55 (CCMi002BMD-A-9 Δ 45–55).

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Resource table.

Unique stem cell line identifier	CCMi002BMD-A-9 Δ 45–55
Alternative name(s) of stem cell line	BMD1 c.9
Institution	Centro Cardiologico Monzino-IRCCS
Contact information of distributor	Aoife Gowran, agowran@ccfm.it
Type of cell line	iPSC
Origin	Human
Additional origin info	Age: 26 years Sex: Male Ethnicity if known: Caucasian
Cell source	Dermal fibroblasts
Clonality	Clonal
Method of reprogramming	Electroporation with episomal vectors containing the reprogramming factors: hL-MYC, hLIN28, hSOX2, KLF4, hOCT4.
Associated disease	Becker muscular dystrophy
Gene/locus	DMD gene, Xp21.2-p21.1
Method of modification	None
Gene correction	NO

(continued)

Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	19/12/17
Cell line repository/bank	N/A
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.

Resource utility

This iPSC line will enrich knowledge of disease mechanisms underlying dystrophinopathies and aid the screening of novel therapeutics.

Resource details

Becker muscular dystrophy (BMD) is a neuromuscular x-linked disorder which arises due to mutations in the dystrophin gene. BMD occurs

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Table 1
CCMi002BMD-A-9 Δ 45-55 summary information, characterisation and quality control analyses.

Classification	Test	Result	Data
Morphology	Photography	Normal morphology.	Fig. 1A
Phenotype	Immunocytochemistry	Assess expression of the pluripotency markers: SSEA4 and Tra-1-60.	Fig. 1B
	Flow cytometry	Expression of the pluripotent cell surface marker Tra-1-60: 75% +ve	Fig. 1C
Genotype	Karyotype (Q-banding) and resolution	e.g. 46XY. Resolution: 400 band level	Fig. 1E
Identity	Microsatellite PCR (mPCR)	Not performed	–
	STR analysis and match	e.g. 17 STR loci plus gender determining locus and matched against the ATCC STR database.	Supplementary material
Mutation analysis (if applicable)	Sequencing	Not performed	–
	MLPA	Deletion of exons 45 to 55 from the DMD gene.	Fig. 1F
Microbiology and virology	Mycoplasma	Mycoplasma testing by RT-PCR: Negative.	Supplementary material Fig. S1
Differentiation potential	Directed differentiation	Line expressed markers for each of the three germ layers: Nestin, ectoderm; α SMA, mesoderm, and 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	–

at one-third the incidence rate of Duchenne muscular dystrophy (DMD; 1:3802–1:6291 male births; Flanigan, 2012). BMD has a wide clinical spectrum, from asymptomatic patients to those restricted to a wheelchair by age sixteen. Although the majority of patients survive to late adulthood (50–60 years), many develop cardiomyopathy characterised by a progressive decline in ejection fraction and early onset heart failure. Certain exons of the dystrophin gene are more indispensable than others for determining phenotype severity. BMD mutations preserve the dystrophin reading frame thus permitting some degree of expression, albeit as truncated isoforms (Muntoni et al., 2003).

Following ethical approval and patient informed consent dermal fibroblasts were isolated by explant culture of a skin biopsy obtained from a 26-year-old man with BMD. To protect privacy, no identifying patient information is included. The patient presented with an incidental finding of hyperkalemia at 15 years old. At time of biopsy the patient was ambulant, however displayed reduced proximal strength in the upper and lower limbs, and exertion-induced myalgia. The Becker patient's fibroblasts were reprogrammed into induced pluripotent stem cells (iPSCs); (Table 1) by electroporation with plasmids encoding L-MYC, LIN28, SOX2, KLF4, OCT4 (Okita et al., 2007) under feeder-free defined conditions. After 28 days of reprogramming, iPSC colonies were selected and expanded. The iPSC line described here was named CCMi002BMD-A-9 and entered further expansion and characterisation for: pluripotent cell morphology (Fig. 1A), expression of pluripotency proteins (SSEA4 and Tra-1-60; Fig. 1B) by immunofluorescence. CCMi002BMD-A-9 were 66% Tra-1-60 positive (Fig. 1C) as assessed by FACS with no significant differences compared to iPSC lines obtained from healthy fibroblasts (88% Tra-1-60 positive). CCMi002BMD-A-9 differentiated into all three germ layers *in vitro* as indicated by immunocytochemistry showing expression of the ectodermal marker nestin; the mesodermal marker alpha-smooth muscle actin (α -SMA), and the endodermal marker Sox17 (Fig. 1D). CCMi002BMD-A-9 was karyotypically normal at P15 and P21 as determined by the analysis of 30 metaphases which showed normal size, shape, and number of chromosomes (Fig. 1E). The identity of CCMi002BMD-A-9 was confirmed by multiple ligation probe amplification (MLPA) of DNA extracted from CCMi002BMD-A-9 which was compared to a control iPSC line (derived from a healthy control subject's dermal fibroblasts). Maintenance of the

DMD reading frame was confirmed by the presence of a large deletion of exons 45 to 55 (CCMi002BMD-A-9 Δ 45-55; Fig. 1F).

Materials and methods

Reprogramming of BMD 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-hSK, pCXLE-hOCT4; 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% CO₂. On day 3 post transfection transfected fibroblasts were transferred to a reprogramming media (ReproTeSR™, Stemcell Technologies) which was replaced every day. Colonies were harvested when they reached 1000 μ m 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.

Pluripotency marker immunocytochemistry

For the analysis of pluripotency protein expression, SSEA4 and Tra-1-60 were detected using commercially available antibodies. iPSCs were analysed with a confocal microscope (Zeiss LSM710). All primary and secondary antibody details are listed in Table 2.

Fig. 1. Derivation of the Becker muscular dystrophy induced pluripotent stem cell line CCMi002BMD-A-9 Δ 45-55. **A** Representative transmitted light images showing the pluripotent cell morphology. **B** Expression of SSEA4 and Tra-1-60 pluripotency-associated markers in CCMi002BMD-A-9 Δ 45-55 was determined by immunofluorescence staining. **C** FACS analysis showed large percentages of cells in CCMi002BMD-A-9 Δ 45-55 and a Control iPSC line expressed the pluripotency marker Tra-1-60 as indicated above by the summary data graph and a representative FACS analysis histogram (Control v CCMi002BMD-A-9 Δ 45-55, values are represented as mean \pm SEM, Student's t test, n = 3). **D** An *in vitro* trilineage differentiation assay revealed that CCMi002BMD-A-9 Δ 45-55 was capable of differentiation into all three germ layers. **E** Karyogram from CCMi002BMD-A-9 Δ 45-55 displaying a normal 46, XY karyotype without any measurable anomalies. **F** Multiplex ligation-dependent probe amplification assay (MLPA) revealed a normal DMD genotype for Control iPSCs (lower electropherograms) while CCMi002BMD-A-9 Δ 45-55 presented deletions of DMD exons from 45 to 55 (upper electropherograms) that is consistent with an in-frame mutation.

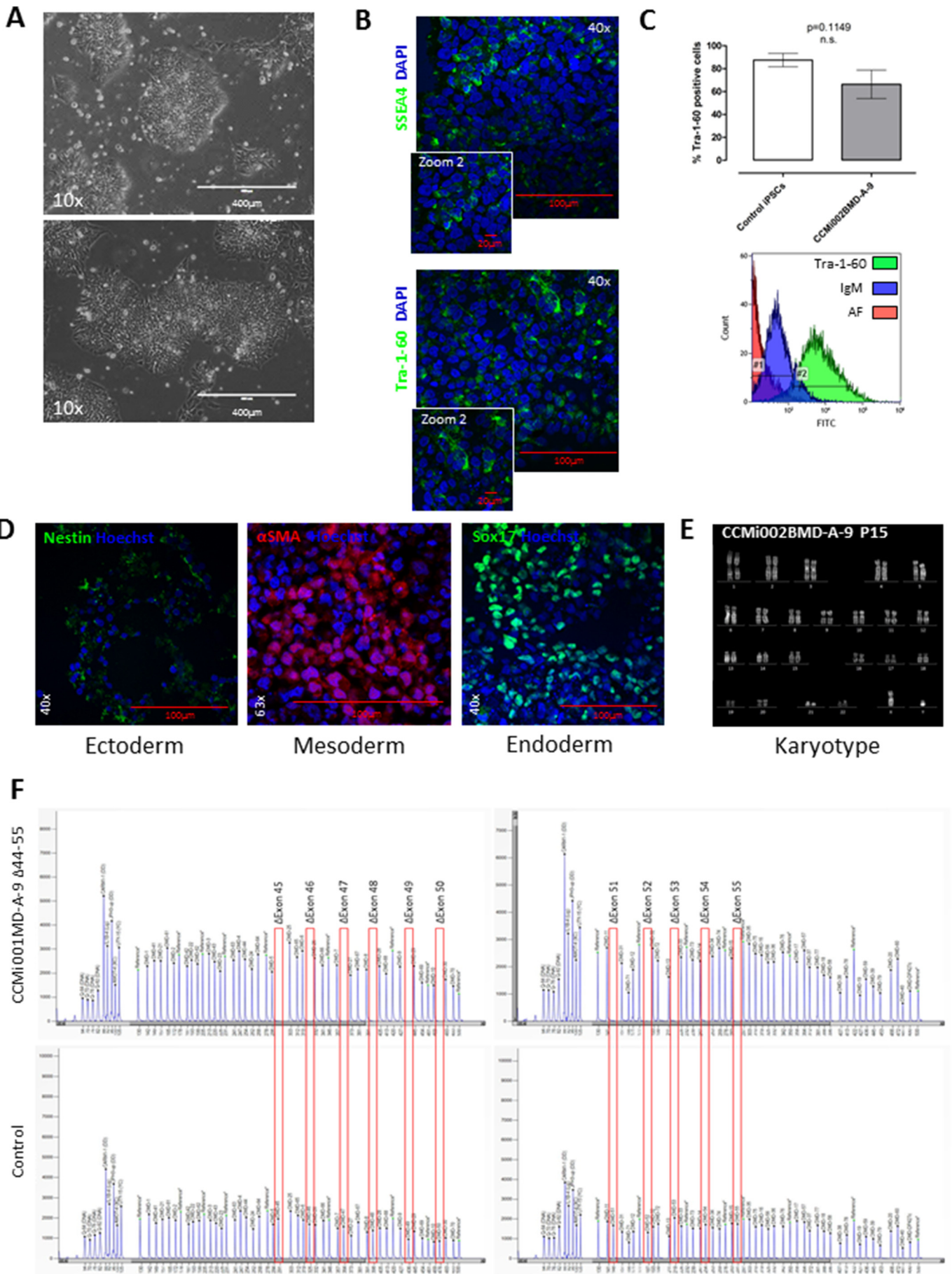


Table 2RRID Requirement for antibodies: use <http://antibodyregistry.org/> to retrieve RRID for antibodies and include ID in table as shown in examples.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Mouse anti-SSEA4	1:200	Abcam Cat# ab16287, RRID:AB_778073;
	Mouse anti-Tra-1-60	1:200 (IFC) 1:100 (FACS)	Abcam Cat# ab16288 RRID: AB_ AB_778563;
Differentiation markers (DM)	Mouse anti-Nestin	1:100	Stemcell Technologies Cat# 60091AD, RRID:AB_2650581;
	Rabbit anti-Sox17	1:100	Cell Signaling Inc. Cat#81778, RRID:AB_2650582;
	Mouse anti- α SMA	1:100	Millipore Cat# CBL171, RRID:AB_2223166;
Secondary antibodies	Anti-Mouse IgG, Alexa@Fluor 488	1:400 (for SSEA4)	Thermo Fisher Scientific Cat# A11059, RRID:AB_2534106;
	Anti-Rabbit IgG, Alexa@Fluor 488	1:200 (for Sox17)	Thermo Fisher Scientific Cat# A11034, RRID:AB_2576217;
	Anti-Mouse IgM, Alexa@Fluor 488	1:400 (for Tra-1-60)	Thermo Fisher Scientific Cat# A21042, RRID:AB_ AB_2535711;
	Anti-Mouse IgG2a Cross-Adsorbed Secondary Antibody, Alexa@Fluor 633	1:200 (for α SMA)	Thermo Fisher Scientific Cat# A-21136, RRID:AB_2535775;

Flow cytometry

CCMi002BMD-A-9 Δ 45-55 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 Tra-1-60 (1:100, 1 h; ab16288 Abcam) per reaction followed by goat anti-mouse IgM-FITC (1:200, 1 h; A21042 Life Technologies). Cells were analysed using a FACSCalibur™ (BD Biosciences) and Gallios (Beckman Coulter) flow cytometers.

In vitro trilineage differentiation potential assay

CCMi002BMD-A-9 Δ 45-55 iPSCs were cultured and differentiated as per the manufacturer's instructions (STEMdiff™ trilineage differentiation kit, Stemcell Technologies). All primary and secondary antibody details are listed in Table 2.

Karyotyping

Metaphase chromosomes were prepared from CCMi002BMD-A-9 Δ 45-55 cultures at passage 15 and 21. After 48–96 h colcemid (10 μ g/ml) was added directly to the cultures and incubated for 3 h at 37 °C. iPSCs were incubated in hypotonic solution (Sodium Citrate 0.6%, KCl

0.13%) at room temperature 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 \times magnification (Immersion oil: Immersol 518N, Zeiss) using a Olympus BX microscope connected to a U-CMAD3 Olympus camera. 30 metaphases were analysed and karyotyped using an automated cytogenetic imaging system (MetaSystems GmbH, Germany).

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2018.01.025>.

Acknowledgements

We thank for Dr. Viviana Meraviglia technical assistance in fibroblast isolation from dystrophic patients' skin biopsies.

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