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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 Δ45-55*)

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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 Δ45-55).



Resource table.

| | |
|---|---|
| Unique stem cell line identifier | *CCMi002BMD-A-9 Δ45-55* |
| 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*<br>*Sex: Male*<br>*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* |
| 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 Δ45-55 summary information, chracterisation and quality control analyses.

| Classification | Test | Result | Data |
|---|---|---|---|
| Morphology | Photography | *Normal morphology.* | *Fig. 1A* |
| Phenotype | Immunocytochemisty | *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 Fig. S1* |
| Differentiation potential | Directed differentiation | *Line expressed markers for each of the three germlayers: Nestin, ectoderm; αSMA, 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 Δ45-55; 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 precoated 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 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.

### *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 Δ45-55. **A** Representative transmitted light images showing the pluripotent cell morphology. **B** Expression of SSEA4 and Tra-1-60 pluripotency-associated markers inCCMi002BMD-A-9 Δ45-55 was determined by immunofluorescence staining. **C** FACS analysis showed large percentages of cells in CCMi002BMD-A-9 Δ45-55 and a Control iPSCline 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 Δ45-55, values are represented as mean ± SEM, Student's t test, n = 3). **D** An in vitro trilineage differentiation assay revealed that CCMi002BMD-A-9 Δ45-55 was capable of differentiation into all three germlayers. **E** Karyogram from CCMi002BMD-A-9 Δ45-55 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 Δ45-55 presented deletions of *DMD* exons from 45 to 55 (upper electropherograms) that is consistent with an in-frame mutation.

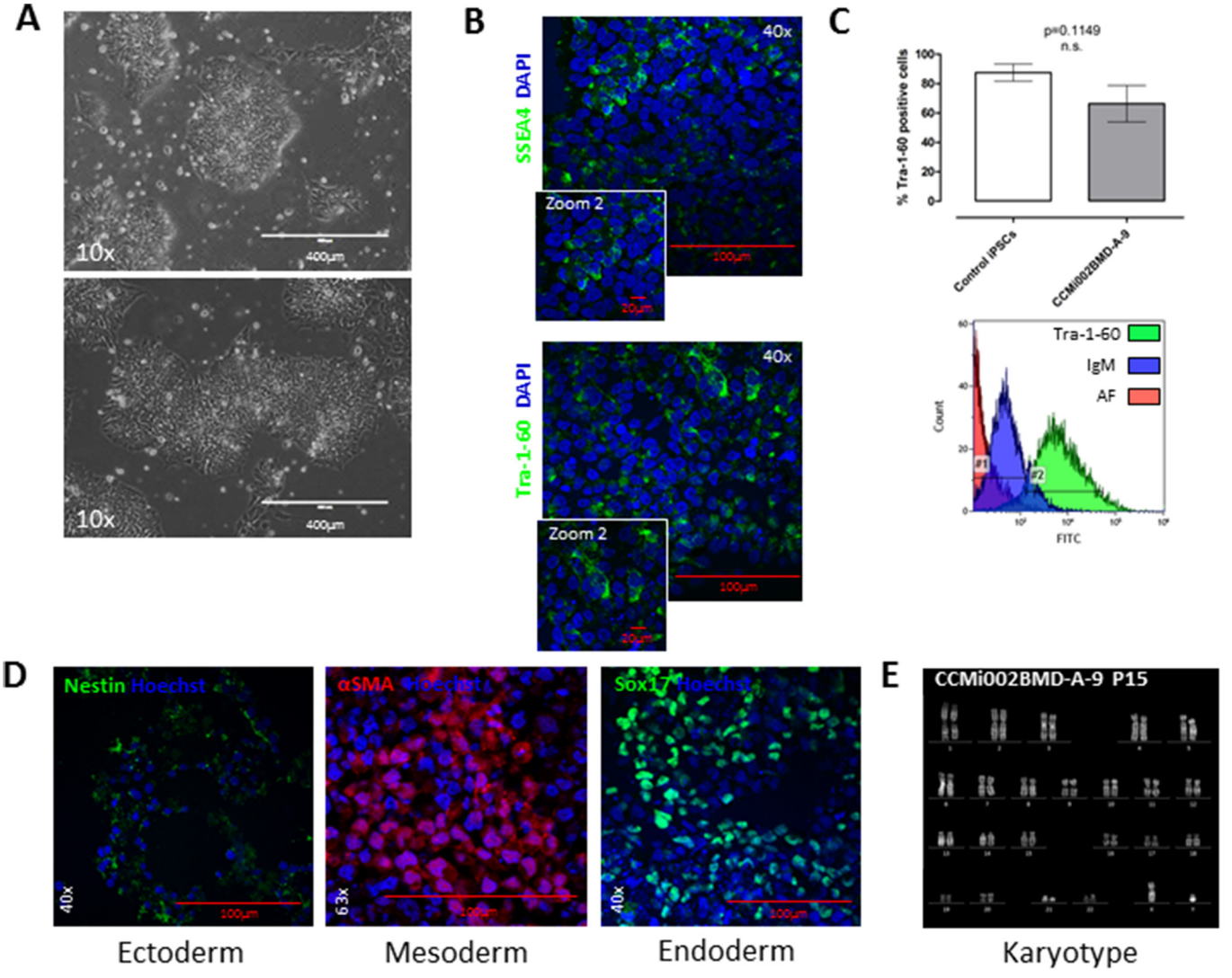
A
10x
400µm
10x
400µm
B
SSEA4 DAPI
40x
Zoom 2
100µm
20µm
Tra-1-60 DAPI
40x
Zoom 2
100µm
20µm
C
p=0.1149
n.s.
% Tra-1-60 positive cells
100
80
60
40
20
0
Control iPSCs
CCMi002BMD-A-9
Tra-1-60
IgM
AF
Count
#1
#2
FITC
D
Nestin Hoechst
40x
100µm
αSMA Hoechst
63x
100µm
Sox17 Hoechst
40x
100µm
Ectoderm
Mesoderm
Endoderm
E
CCMi002BMD-A-9 P15
Karyotype
F
CCMi001MD-A-9 Δ44-55
Control
ΔExon 45
ΔExon 46
ΔExon 47
ΔExon 48
ΔExon 49
ΔExon 50
ΔExon 51
ΔExon 52
ΔExon 53
ΔExon 54
ΔExon 55

**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* | *Abcam Cat# ab16287, RRID:AB_778073;* |
| | *Mouse anti-Tra-1-60* | *1:200 (IFC)* | *Abcam Cat# ab16288* |
| | | *1:100 (FACS)* | *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-αSMA* | *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 αSMA)* | *Thermo Fisher Scientific Cat# A-21136, RRID:AB_2535775;* |

*Flow cytometry*

CCMi002BMD-A-9 Δ45-55 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 Δ45-55 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 Δ45–55 cultures at passage 15 and 21. After 48–96 h colcemid (10 μg/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× 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.

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