Stem Cell Research 45 (2020) 101819

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Lab resource: Stem Cell Line

Generation of the Becker muscular dystrophy patient derived induced pluripotent stem cell line carrying the *DMD* splicing mutation c.1705-8 $T>C$.

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A B S T R A C T

Becker Muscular dystrophy (BMD) is an X-linked syndrome characterized by progressive muscle weakness. BMD is generally less severe than Duchenne Muscular Dystrophy. BMD is caused by mutations in the dystrophin gene that normally give rise to the production of a truncated but partially functional dystrophin protein. We generated an induced pluripotent cell line from dermal fibroblasts of a BMD patient carrying a splice mutation in the dystrophin gene (c.1705-8 $T>C$). The iPSC cell-line displayed the characteristic pluripotent-like morphology, expressed pluripotency markers, differentiated into cells of the three germ layers and had a normal karyotype.

Resource Table:

Unique stem cell line identifier	CCMi004-A
Alternative name(s) of stem cell line	BMD3 c.13
Institution	*Centro Cardiologico Monzino-IRCCS*
Contact information of distributor	*Aoife Gowran; aoife.gowran@ccfm.it*
Type of cell line	*iPSC*
Origin	*Human*
Additional origin info	*Age: 5 (at skin biopsy)* *Sex: M* *Ethnicity if known: Caucasian*
Cell Source	*Dermal fibroblasts*
Clonality	*Clonal*
Method of reprogramming	*Episomal vectors containing the reprogramming factors: hL-MYC, hLIN28, hSOX2, hKLF4, hOCT4.*
Genetic Modification	*YES*
Type of Modification	*Spontaneous mutation*
Associated disease	*Becker 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	*July 2019*
Cell line repository/bank	*The Telethon Biobank and the Eurobiobank*

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https://doi.org/10.1016/j.scr.2020.101819
Received 30 January 2020; Received in revised form 20 March 2020; Accepted 12 April 2020
Available online 22 April 2020

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. 20190528 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.*

Resource utility

This cell line provides a tool to investigate novel patho-mechanisms caused by mutations in the dystrophin gene and will help the screening of novel therapeutics.

Resource Details

Becker Muscular Dystrophy (BMD) is an X-linked syndrome characterized by progressive skeletal muscle weakness and the development of cardiomyopathy. BMD is generally less severe than Duchenne Muscular Dystrophy (DMD) the other forms of muscular dystrophy (Flanigan, 2014). BMD is caused by mutations in the dystrophin (*DMD*) gene, that codes for a structural protein localized under the sarcolemma. The dystrophin protein links the actin cytoskeleton to the extracellular matrix *via* the dystrophin-associated protein complex (DAPC) (Constantin, 2014). BMD mutations preserve the dystrophin reading frame allowing the expression of a truncated but partially functional dystrophin (Muntoni et al., 2003). There is significant phenotypic heterogeneity in BMD, 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 characterized by a progressive decline in ejection fraction and heart failure.

After obtaining informed consent and institutional ethical approval dermal fibroblasts were isolated from a 5-year-old child with BMD. To protect privacy, no identifying patient information is included. The subject presented with incidental HyperCKemia *i.e.* abnormal levels of creatine kinase (CK) in the blood. CK is released into the circulation as a consequence of deteriorating myofibrils, which is caused by the lack of mechanical stabilization of the sarcolemma normally provided by functional dystrophin. At time of biopsy the patient was still ambulant. The patient's fibroblasts were electroporated with plasmids encoding L-MYC, LIN28, SOX2, KLF4, OCT4 (Okita et al., 2007) and reprogrammed to induced pluripotent stem cells (iPSCs) under feeder-free conditions.

The iPSC cell-line here described, named CCMi004-A, was characterized for iPSC pluripotent cell morphology (Fig. 1A) and expression of the pluripotency marker SSEA4 by immunofluorescence (Fig. 1B). The majority of CCMi004-A cells (81%) were positive for the presence of SSEA4 as demonstrated by FACS experiments, with no significant difference compared to well-characterized iPSC cell lines derived from healthy individuals (Fig. 1C).

In vitro trilineage differentiation assays showed that CCMi004-A is able to differentiate into cells of each germ layer (Ectoderm NESTIN/PAX6, mesoderm cardiac troponin T type 2 CTNT2 and endoderm SOX17; Fig. 1D). Karyotype analyses, performed on more than 30 metaphases, demonstrated that the iPSC line has a normal karyotype (Fig. 1E). Sanger sequencing, performed on genomic DNA extracted from CCMi004-A showed the presence of the single nucleotide substitution $T>C$ in the intronic region spanning exons 14 and 15 (c.1705–8 $T>C$) (Fig. 1F). This mutation alters the splicing of exon 15 allowing the production of a shorter mRNA. Short Tandem Repeat (STR) analysis of 17 total loci (LGC Standards and ATCC) showed that CCMi004-A iPSCs were unique (no match to any other cell line in the ATCC-STR database or DSMZ) and matched the patient's primary fibroblasts. Mycoplasma-free CCMi004-A (Supplementary Figure 1) were harvested and frozen in mFreSR™ (Stemcell Technologies) and transferred to long-term nitrogen storage.

Materials and methods

Reprogramming of BMD patient's fibroblasts to iPSCs

The fibroblasts were isolated from the patient's skin biopsy by explant culture. Fibroblasts were transfected with 1.25 μg of episomal vectors (pCXLE-hUL, pCXLE-hSK, pCXLE-hOCT4; Addgene) by electroporation (1650 V, 10 s, 3 pulse; Neon™ transfection system, Invitrogen), transferred into a single well of a 6-well plate coated with human recombinant vitronectin (Life Technologies) and cultured at 37 °C with 5% CO_2. On day 3 post transfection transfected fibroblasts were cultured with 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. Every 3–4 days, iPSCs were non-enzymatically passaged 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).

Pluripotency marker immunocytochemistry

CCMi004-A were cultured in vitronectin-coated chamber slides for analysis of pluripotency proteins (SSEA4). iPSCs were fixed in 4% formaldehyde (10 min RT), 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. Slides were analysed with a confocal microscope (LSM710, Zeiss). All antibody details are listed in Table 2.

Flow cytometry

iPSCs detached using ReLeSR™ (Stemcell Technologies), were resuspended in PBS/0.5 mM EDTA, fixed for 20 min on ice using 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). All the antibodies were diluted in 0.1% BSA, 0.5 mM EDTA in PBS. iPSCs were analysed using a Gallios (Beckman Coulter) flow cytometer and Kaluza software (version 2.5, Beckman Coulter). iPSC lines from healthy donors were used as characterization controls.

In vitro trilineage differentiation potential assay

CCMi004-A cells were differentiated into cells of the ectodermal or endodermal lineages using the STEMdiff™ trilineage differentiation kit (Stemcell Technologies) according to the manufacturer's instructions. For differentiation along the mesodermal lineage, cells 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 and analysed by confocal microscopy (LSM710, Zeiss). All antibody details are listed in Table 2.

Karyotyping

Metaphase chromosomes were prepared from iPSC cultures at passage 25 (P25). 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 with methanol/acetic acid (3:1) in Optichrome (28 °C, 42% rH), Q-banded and photographed. Karyotype images were

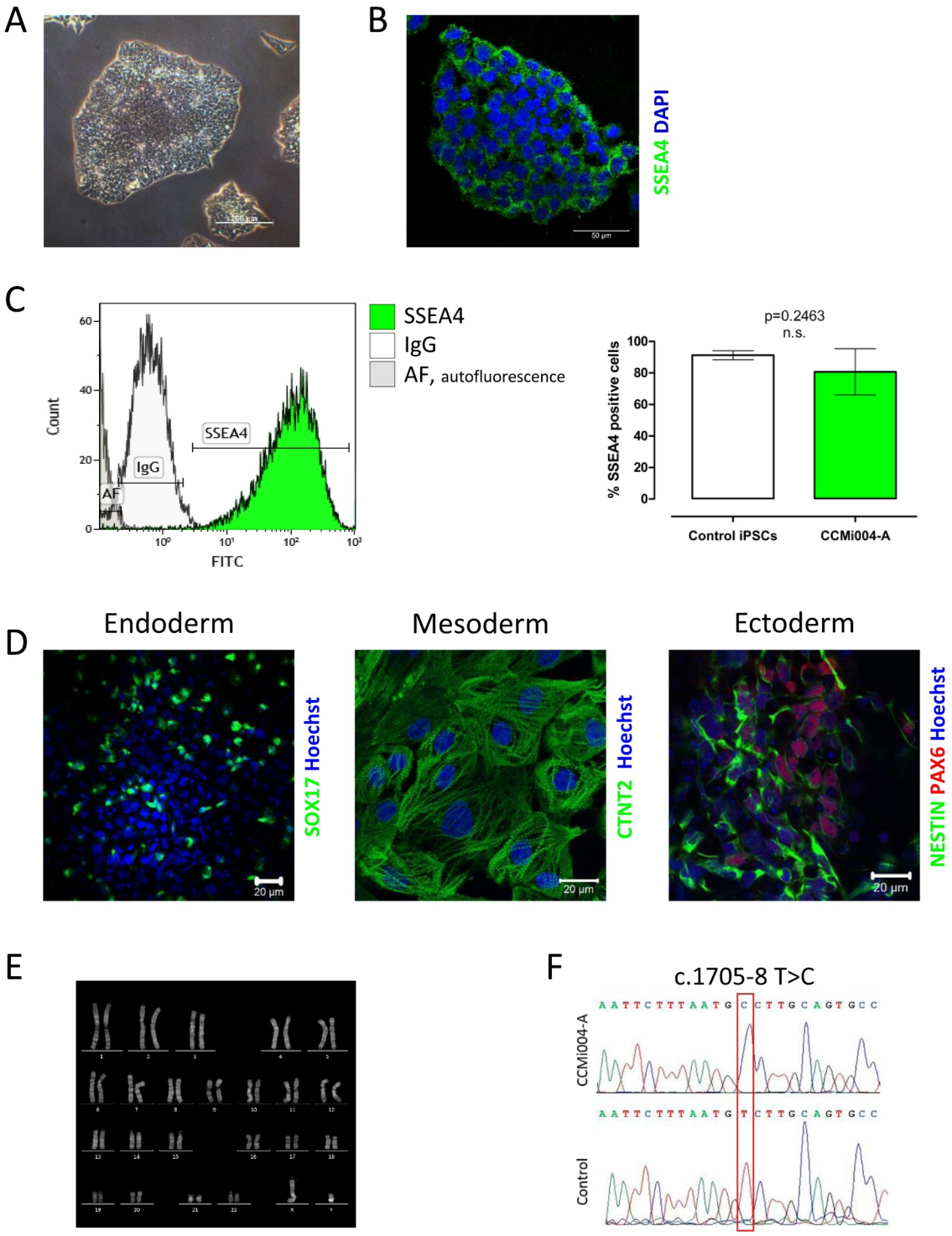

Fig. 1. Characterization of Becker Muscular Dystrophy induced pluripotent stem cell line (CCMi004-A).

obtained at 100X magnification (Olympus BX microscope, U-CMAD3 Olympus camera). About 30 metaphases were analysed and karyotyped using an automated cytogenetic imaging system (MetaSystems Gmbh, Germany).

Dystrophin mutation analyses by Sanger sequencing

DNA was extracted from iPSCs using the QIAamp DNA Mini Kit (Qiagen) according to manufacturer's instructions. The *DMD* intron 14–15 was amplified with GoTaq Flexi DNA polymerase (Promega) using exon flanking primers (95 °C-56 °C-72 °C, 35 cycles). PCR products were then sent to Microsynth for direct Sanger sequencing. Electropherograms were aligned and analysed with ChromasPro software (Technelysium Pty Ltd). DNA extracted from iPSCs obtained from a healthy individual's dermal fibroblasts were used as controls.

STR analysis

STR analysis was performed by the ATCC cell-line authentication service. Seventeen STR loci plus the gender-determining locus,

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 (81%)*	*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 loci test, all matched*	*submitted in archive with journal*
Mutation analysis (IF APPLICABLE)	Sequencing	*DMD mutation c.1705-8 T>C*	*Fig. 1F*
	Southern Blot OR WGS	*Not performed*	
Microbiology and virology	Mycoplasma	*Mycoplasma testing by PCR; Negative*	*Supplementary figure 1*
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*	

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;&&&Thermo Fisher Scientific Cat# MA-512960, RRID: AB_11000742;&&&Abcam Cat# ab22035, RRID: AB_446723&&&BioLegend Cat# PRB-278P, RRID: AB_291612*
	Mouse anti-CTNT2	*1:300*	
	Mouse anti-NESTIN	*1:150*	
	Rabbit anti-PAX6	*1:300*	
Secondary antibodies	*Anti-Mouse IgG, Alexa®Fluor 488 Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa®Fluor 594*	*1:400 (for SSEA4) 1:300 (for NESTIN) 1:200(for CTNT2) 1:300 (for PAX6)*	*Thermo Fisher Scientific Cat# A11059, RRID:AB_2534106;&&&Thermo Fisher Scientific Cat# A-11037&&&RRID: AB_2534095*
Primers			
	Target	**Forward/Reverse primer (5′ − 3′)**	
Genotyping	*DMD intron14-15 c.1705-8 T>C*	F: CCGCTGGGTTCTTTTACAAG R: AGCCAGTTGTGTGAATCTTGT	

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 verify the absence 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.

Declaration of Competing Interest

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

Acknowledgments

Fondazione IEO-CCM (Pompilio, Rovina), Italian Ministry of Health (Pompilio, Torrente), Fondazione Umberto Veronesi (Gowran), Fondazione Telethon (Pompilio, Torrente) and the European Research Area Network on Cardiovascular Diseases (Gowran).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2020.101819.

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